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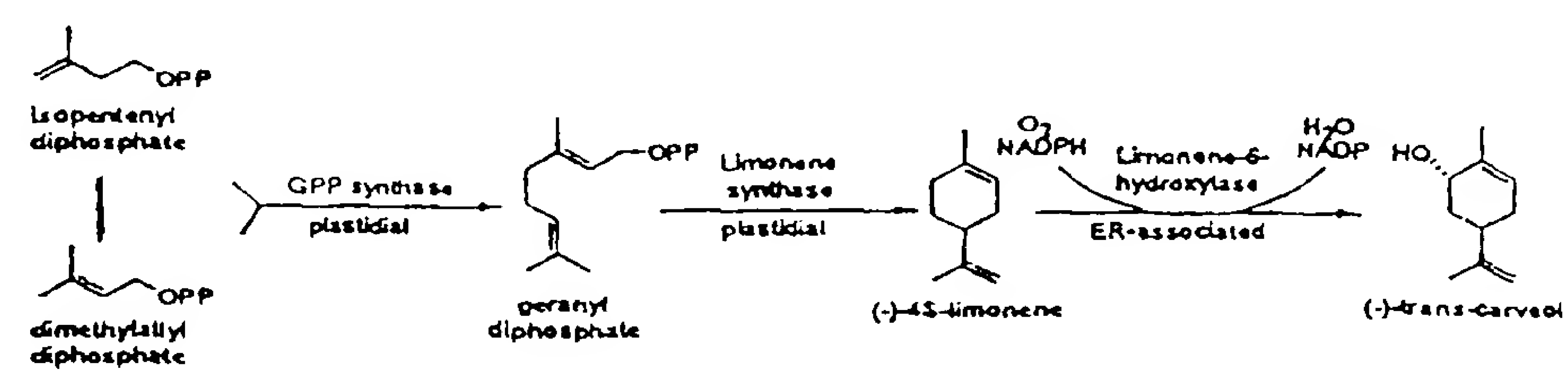

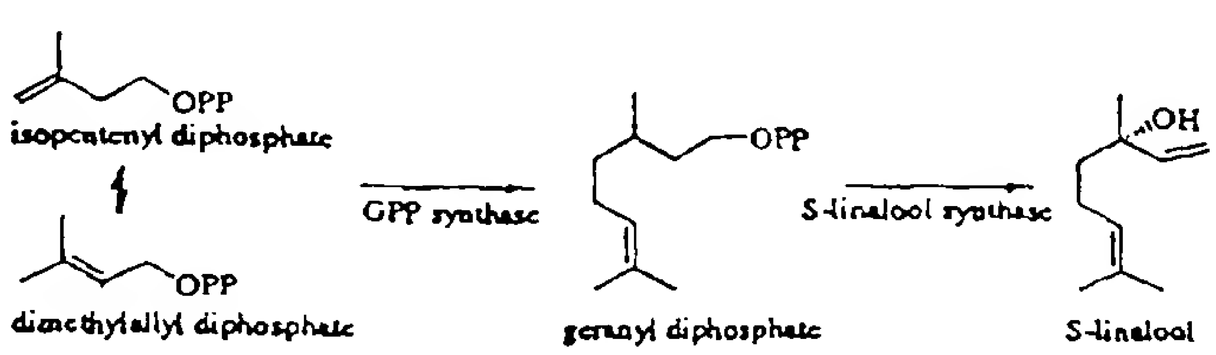

(57) Abstract

Methods for manipulating metabolic pathway in plants, particularly those pathways involved in the biosynthesis of monoterpenes are provided. Methods are directed at transforming plants with one or more nucleotide sequences encoding the enzyme GPP synthase, and the monoterpene synthases limonene-, carveol and S-linolool synthase. Methods for creating or enhancing resistance to insects in plants by transforming plants with GPP- and/or monoterpene synthases, to generate plants producing monoterpenes in amount effective for resistance to insects are also provided.

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LIMONENE AND OTHER DOWNSTREAM METABOLITES OF GERANYL PYROPHOSPHATE FOR INSECT CONTROL IN PLANTS

FIELD OF THE INVENTION

This invention relates to methods for genetic manipulation of metabolic pathways in plants, particularly to transforming plants with genes involved in monoterpene biosynthesis and resistance to insects.

BACKGROUND OF THE INVENTION

Numerous insects are serious pests of common agricultural crops. One method of controlling insects has been to apply insecticidal organic, semiorganic or organometallic chemicals to crops. This method has numerous, art-recognized environmental and public health problems. A more recent method of control of insect pests has been the use of biological control organisms which are typically natural predators of the troublesome insects. These include other insects such as trachonid wasps, fungi such as *Beauveria Bassiana*, and bacteria such as Bacillus thuringiensis cv., commonly referred to as "Bt". However, it is difficult to apply biological control organisms to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period. Still more recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express insecticidal toxins derived from biological control organisms such as Bt. This technology has given rise to concerns about eventual insect resistance to well-known, naturally occurring insect toxins, particularly in the face of heavy selection pressure, which may occur in some areas. Thus, a continuing need exists to identify naturally occurring insecticidal toxins which can be formed by plant cells directly by expression of structural genes not normally present in the plant.

Southern Corn Rootworm (*Diabrotica undecimpunctata howardi* Barber) is a particularly difficult pest to control or eradicate. It attacks the plant below the soil line, where insecticides are difficult or impossible to apply effectively. In addition, it is resistant to a number of otherwise effective chemical and biological control agents, including Bt toxins and some lectins.

The monoterpene, limonene, 1-methyl-4 (1-methylethenyl) cyclohexene, p-mentha-1,8-diene (Entry No. 5371, Merck Index 11th Ed.), occurs naturally in various ethereal oils, particularly oils of lemon, orange, caraway, dill and bergamot. It is a valuable industrial chemical. Some limonene is prepared by extraction from plants of the mint family, a large quantity is obtained from citrus oils, which are typically 80-90% limonene, and some is obtained from pine oil. It is also synthesized chemically and finds use as a solvent and cleaning agent (in the manufacture of synthetic pine oil), as an expectorant, as a wetting and dispersing agent, as a monomer in the manufacture of various polymeric resins, as a flavorant and a precursor in the synthesis of the flavorant carvone, and as a polymerization inhibitor in storage of the tetrafluoreoethylene monomer used in the manufacture of polytetrafluoroethylene (PTFE).

Geranyl diphosphate (GPP) synthase catalyzes the first committed step of monoterpene biosynthesis by the condensation of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) to form GPP, the immediate acyclic precursor of monoterpenes. GPP is converted to (-)-4S-limonene by the catalytic action of (-)-4S-limonene synthase (cyclase). (-)-4S-limonene is converted to (-)-trans-carveol by the action of (-)-trans-carveol synthase, also referred to as limonene-6-hydroxylase. -(-)trans-carveol is converted to carvone by the action of -(-)trans-carveol dehydrogenase, also referred to as carvone synthase. GPP is also converted to the monoterpene S-linalool by the action of S-linalool synthase. Thus, GPP is the precursor of (-)-4S-limonene and its downstream metabolites (-)-trans-carveol and carvone; as well as the precursor of S-linalool. See Figure 1; Wise *et al.* (1997) In "*Comprehensive Natural Products Chemistry: Isoprenoids, Vol. 2*" (Cane, D.E., ed.), Elsevier Science, Oxford (in press); Gershenzon *et al.* (1989) *Plant Physiol. 89*:1351-1357; Pichersky *et al.* (1994) *Plant Physiol. 106*:1533-1540. Unlike the mechanistically-related prenyltransferases farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase, which produce GPP as intermediates and which are nearly ubiquitous (Ogura *et al.* (1997) In "*Dynamic Aspects of Natural Products Chemistry*" (Ogura, K. and Sankawa, U., eds.), Kodansha/Harwood Academic Publishers, Tokyo, pp. 1-23), GPP synthase is largely restricted to plant species that produce abundant quantities of monoterpenes.

Because both farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase produce only negligible levels of GPP as a free intermediate on route to FPP and GGPP (Ogura *et al.* (1997) In "*Dynamic Aspects of Natural Products Chemistry*" (Ogura, K. and Sankawa, U., eds.), Kodansha/Harwood Academic Publishers, Tokyo, pp. 1-23), it is geranyl diphosphate synthase that provides the crucial link between primary metabolism and monoterpene biosynthesis and that serves as the essential driver of monoterpene biosynthesis (Wise *et al.* (1997) In "*Comprehensive Natural Products Chemistry: Isoprenoids, Vol. 2*" (Cane, D.E., ed.), Elsevier Science, Oxford (in press)).

GPP synthase has been isolated from several plant sources, including grape, geranium, sage (Croteau *et al.* (1989) *Arch. Biochem. Biophys. 271*:524-535; Heide *et al.* (1989) *Arch. Biochem. Biophys. 273*:331-338; Suga *et al.* (1991) *Phytochemistry 30*:1757-1761; Clastre *et al.* (1993) *Plant Physiol. 102*:205-211); however, the enzyme has not been characterized in any detail, and only the enzyme from grape has been purified to homogeneity.

A cDNA encoding 4S-limonene synthase from oil glands of spearmint has been described in Colby *et al.* (1993) *J. Biol. Chem. 268(31)*: 23016-23024 and is available in the Genbank™/EMBL database and identified by the accession number L13459.

cDNAs encoding S-linalool synthase from *Clarkia Breweri* have been described in WO 97/15584, along with methods directed at using the cDNA for enhancing the flavor and smell of plants; and in Dudareva *et al.* (1996) *Plant Cell 8 (7)*:1137-1148, also available in the Genbank™/EMBL database and identified by the accession number 1491939.

To exploit recombinant methods to increase monoterpene yield in monoterpene-producing species, or to genetically engineer the monoterpene biosynthetic pathway into non-producing species, it would be highly beneficial to manipulate a GPP synthase gene. Accordingly, the invention relates to expressing GPP synthase in combination with selected monoterpene synthases such as (-)-limonene synthase, S-linalool synthase, and subsequent and related pathway enzymes for production of the corresponding monoterpene product(s).

SUMMARY OF THE INVENTION

The invention provides methods for manipulating metabolic pathways in plants, particularly those pathways that are involved in the biosynthesis of monoterpenes. Methods are directed at transforming plants, plant tissues and cells with one or more nucleotide sequences encoding the enzyme GPP synthase, and the monoterpene synthases limonene-, carveol and S-linolool synthase.

Methods are also provided for creating or enhancing resistance to insects in plants by transforming plants with GPP- and/or monoterpene synthases, to generate plants producing monoterpenes in amounts effective for resistance to insects.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the biosynthetic pathways involved in production of the monoterpenes limonene, carveol, and S-linalool.

Figure 2 schematically illustrates the plasmid construct comprising the ubiquitin promoter and a monoterpene synthesis pathway enzyme.

Figure 3 schematically illustrates the plasmid construct PHP14099 for expression of GPP- and S-linalool synthases, and production of S-linalool in plants.

Figure 4 schematically illustrates the plasmid construct PHP14100 for expression of GPP-, limonene- and carveol synthases; and production of limonene and carveol.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for manipulating metabolic pathways in plant cells; particularly those pathways related to synthesis of monoterpenes including but not limited to the monoterpenes (-)-4S-limonene (herein limonene), (-)-trans-carveol (herein carveol) and S-linalool in plants, plant cells and specific plant tissues. These monoterpenes have insecticidal and/or repellent activity against insect pests.

Accordingly, the invention provides methods useful for increasing monoterpene yield in monoterpene-producing species such as mint, and for producing monoterpenes in species which typically do not produce monoterpenes such as maize. The invention also provides methods for creating or enhancing resistance to insects in plants by transforming plants with nucleotide sequences encoding monoterpene synthesis pathway enzymes and generating transformed plants which produce effective amounts of desired monoterpenes in the plant. In this aspect, by "effective amount" is intended that amount of a monoterpene, alone or in combination with other agents, that can effect a reduction, amelioration, prevention, or elimination of a plant-insect interaction.

Thus, the methods of the invention are directed at transforming plant cells with at least one nucleotide sequence encoding a monoterpene synthesis pathway enzyme selected from GPP synthase, limonene synthase, carveol synthase, S-linalool synthase, or combinations thereof.

By "monoterpene synthase" is intended an enzyme which catalyzes a reaction having at least one monoterpene as the product of the reaction. More specifically, monoterpene synthases utilized in the methods of the invention include, but are not limited to limonene synthase, carveol synthase (limonene 6-hydroxylase) and S-linalool synthase.

By "transgenic plant" is meant any plant or plant cell that has become transformed by the introduction, stable and heritable incorporation, into the subject plant or plant cell, of either native DNA that is under the control of a promoter other than the promoter that typically drives expression of that DNA in a wild-type plant, and that has been introduced back into its host plant, or foreign DNA, i.e. DNA encoding for a protein not normally found within that plant species.

"Plantlet" refers to a plant sufficiently developed to have a shoot and a root that is asexually reproduced by cell culture.

"Explant" refers to a section or piece of tissue from any part of a plant for culturing.

The term "callus" and its plural "calli", refer to an unorganized group of cells formed in response to cutting, severing, or other injury inflicted on plant tissue. Excised pieces of plant tissue and isolated cells can be induced to form callus under the appropriate culture conditions. Callus can be maintained in

culture for a considerable time by transferring or subculturing parts of the callus to fresh medium at regular intervals. The transfer of callus to liquid medium leads to dispersion of the tissue and the formation of a plant cell suspension culture. Callus can be induced to undergo organized development to form shoots and roots.

"Embryoid" refers to a structure similar in appearance to a plant zygotic embryo.

By the term "taxon" herein is meant a unit of botanical classification of genus or lower. It thus includes genus, species, cultivar, variety, variant, and other minor taxonomic groups that lack a consistent nomenclature.

"Somatic hybrid" and "somatic hybridization" refers generally to stable combination of cellular material, be it protoplast/protoplast or protoplast/cytoplast combinations, and includes cybrids and cybridization.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as a unit of DNA replication in vivo; i.e., capable of replication under its own control.

As used herein, the term "nucleotide sequence" means a DNA or RNA sequence, and can include a cDNA, or genomic DNA, or synthetic DNA sequence, a structural gene or a fragment thereof, or an mRNA sequence, that encodes an active or functional polypeptide.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

A "promoter sequence" or a "promoter" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream

(3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at its 3′ terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" or "under the control of" control sequences in a cell when RNA polymerase will bind the promoter sequence and transcribe the mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of undergoing transformation, by an exogenous DNA sequence.

A cell has been "transformed" by endogenous or exogenous DNA when such DNA has been introduced inside the cell membrane. The DNA may or may not be integrated into (covalently linked to) chromosomal DNA making up the genome of the transformed cell. In procaryotes, for example, the DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association

with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacterium. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). "Heterologous" DNA also refers to DNA not found within the host cell in nature. Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as these terms are used herein. "Native", "autologous" or "endogenous" DNA, as used herein, refer to DNA that is typically present in the host in nature.

The term "polypeptide" as used herein is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term " polypeptide" includes proteins, oligopeptides, protein fragments, analogues, muteins, fusion proteins and the like. The term also encompasses amino acid polymers as described above that include additional non-amino acid moieties. Thus, the term "polypeptide" includes glycoproteins, lipoproteins, phosphoproteins, metalloproteins, nucleoproteins, as well as other conjugated proteins. The term "polypeptide" contemplates polypeptides as defined above that are recombinantly produced, isolated from an appropriate source, or synthesized.

By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. Such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance, which then serve as selectable markers to identify and separate transformed cells. Preferred selection agents include kanamycin, chlorosulfuron, phosphinothricin, hygromycin and methotrexate, and preferred markers are genes conferring resistance to these compounds. A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant." A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule that is not a part of the chromosomes of the cell.

In carrying out this invention, it will be appreciated that numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression cassette preferably includes a strong promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the DNA sequence of the present invention can be inserted is the pPHI414 plasmid developed by Beach *et al.* of Pioneer Hi-Bred International, Inc., Johnston, IA. Highly preferred plant expression cassettes are designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

Methods of the invention include those for manipulating a metabolic pathway in a plant cell by transforming with nucleotide sequences for native limonene-, GPP-, carveol- and S-linalool synthase genes, by transforming with nucleotide sequences encoding amino acid sequences for the respective proteins encoded thereby, as well as fragments and variants thereof. Such native sequences are set forth in SEQ ID NOs: 1-8. SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7 set forth the nuceleotide sequences for limonene-, GPP-, carveol-, and S-linalool synthase respectively; the corresponding amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8 respectively. The methods encompass using the sequences or corresponding antisense sequences in modulating the expression of enzymes involved in monoterpene synthesis, as well as production of monoterpenes in a plant or plant cell. That is, the coding sequences are used to increase the expression of an enzyme while antisense sequences are used to decrease expression. In this aspect, blocking the expression of an enzyme within a pathway by antisense sequences can

be used to accumulate the substrates of that enzyme or to drive the pathway to another end product.

It is recognized that the methods of the invention could be used to manipulate metabolic pathways involving reactions downstream of those catalyzed by GPP- and a monoterpene synthase such as limonene-, carveol- and/or S-linalool synthase as described herein; by transforming plants with nucleotide sequences encoding GPP synthase and at least one monoterpene synthase, or antisense sequences thereof. In this aspect, the methods of the invention encompass manipulating the pathway involving production of the compounds including but not limited to (-)-carvone, (-)-trans-isopiperitinol and (-)-trans-isopiperitinone.

Antisense RNA or DNA can be utilized for the accumulation of a particular monoterpene. Alternatively, homologous plant sequences or partial plant sequences can be used. For example, an antisense carveol synthase sequence can be used to cause the accumulation of limonene. In this manner, the metabolic pathway of interest can be manipulated for the high production of any particular monoterpene of interest in the pathway.

Likewise, the pathway can be manipulated to decrease levels of a particular compound by transformation of antisense sequences which prevent the conversion of the precursor compound into the particular compound being regulated. For example, conversion of GPP to S-linalool can be blocked by antisense sequences to S-linalool synthase.

In the same manner, to increase the biosynthesis of a particular desired monoterpene, antisense constructs can be used to block the conversion of a common substrate to one monoterpene, thereby shunting the common substrate to the pathway for the desired monoterpene, while additionally blocking downstream conversion of the desired monoterpene to a further downstream metabolite. For example, an antisense sequence to S-linalool synthase can be used to shunt the common substrate GPP to the pathway for limonene biosynthesis, while additionally antisense sequence to carveol synthase can be used to block conversion of limonene to carveol.

Any means for producing a plant comprising GPP- and at least one monoterpene synthase coding sequence are encompassed by the methods of the present invention. For example, the second (or additional) gene of interest can be

used to transform a plant at the same time as the GPP synthase gene (cotransformation); the second gene can be introduced into a plant that has already been transformed with the GPP synthase gene; GPP synthase can be transformed into a plant has already been transformed with the second gene; or alternatively, transformed plants, one expressing the GPP synthase and one expressing the second gene, can be crossed to bring the genes together in the same plant. Subsequent crosses or transformations can bring additional sequences together in the plant.

The use of fragments and variants of the nucleotide and amino acid sequences are encompassed within the scope of the invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 15 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the entire nucleotide sequence encoding the monoterpene synthase proteins utilized in the methods of the invention. Fragments of the invention include antisense sequences used to decrease expression of the monoterpene synthase genes utilized in the methods of the invention. Such antisense fragments may vary in length ranging from at least about 15 nucleotides, about 50 nucleotides, about 100 nucleotides, up to and including the entire coding sequence.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of the GPP- and carveol synthase genes. Generally, nucleotide sequence variants of the invention will have at least 70%, generally, 80%, preferably up to 90-95% sequence identity to the native nucleotide sequence.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one

or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

For example, amino acid sequence variants of the polypeptide can be prepared by mutations in the DNA sequence encoding the native protein of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods Enzymol. 154*:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

In constructing variants of the limonene-, GPP-, carveol and S-linalool synthase proteins of interest, modifications to the nucleotide sequences encoding the variants will be made such that variants continue to possess the desired activity. Obviously, any mutations made in the DNA encoding the variant protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce excessive secondary mRNA structure. See EP Patent Application Publication No. 75,444.

Thus, nucleotide sequences utilized in the methods of the invention and the proteins encoded thereby include the native forms as well as variants thereof. The variant proteins will be substantially homologous and functionally equivalent to the native proteins. A variant of a native protein is "substantially homologous" to the native protein when at least about 80-85%, more preferably at least about 90%, and most preferably at least about 95% of its amino acid sequence is identical to the amino acid sequence of the native protein. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system.

Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York; Brown, T.A. *Gene Cloning: An Introduction* (2nd Ed.) Chapman & Hall, London (1990).

Alternatively, sequence identity can be determined utilizing a number of available computer programs including but not limited to CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection. For purposes of the present invention, comparison of nucleotide or protein sequences for determination of percent sequence identity to the sequences described herein is preferably made using the Gapped BLAST program (Version 2.0 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

By "functionally equivalent" is intended that the sequence of the variant defines a chain that produces a protein having substantially the same biological effect as the native protein of interest. Such functionally equivalent variants that comprise substantial sequence variations are also encompassed by the invention. Thus, for purposes of the present invention, a functionally equivalent variant of GPP synthase will catalyze the formation of GPP from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP); that of limonene synthase will catalyze the formation of limonene from GPP; that of carveol synthase will catalyze the formation of carveol from limonene; and that of S-linalool synthase will catalyze the formation of S-linalool from GPP.

In view of the ability to transform crop plants to express various heterologous compounds, it would be desirable to transform maize plants to express monoterpene pathway enzymes and thereby produce effective amounts of the monoterpenes limonene, carveol and/or S-linalool so that by consuming the tissues of the plant an insect, such as larvae of Corn Rootworm, would consume insecticidally effective amounts of the monoterpenes; or be caused to avoid feeding on the plant.

While most gene products are peptides, a monoterpene is not a peptide or peptide derivative and is not expressed from genes in the form of a peptide or peptide derivative, but is produced enzymatically as a secondary metabolite within the cells of some plants. It is determined that the biosynthetic apparatus necessary for the production of the monoterpene limonene may not be present in many plant cells which do not produce limonene, or may not produce detectable, insecticidally effective amounts of limonene, and this appears to include maize cells. Such plant cells must be engineered with at least one enzyme which can be produced through the expression of exogenous (heterologous) genes. One such enzyme is limonene synthase, also known as limonene cyclase, which can directly synthesize limonene from geranyl pyprophosphate (GPP), which is found widely in both procaryotic and eucaryotic cells, although, as discussed below, is in some cases not produced in quantities sufficient to make insecticidally effective amounts of limonene.

Since genes which code for a monoterpene synthase can be synthesized, either directly using a DNA sequence obtained by working backwards from the known amino acid sequence of a particular monoterpene synthase and preferably

using plant-preferred codons, or by cloning from natural sources of monoterpenes, the resulting sequence can be inserted into an appropriate expression cassette, and introduced into cells of a susceptible plant species or a suitable endophytic bacterium, so that an especially preferred embodiment of this method involves inserting into the genome of the plant or bacterium a DNA sequence coding for a monoterpene synthase, in proper reading frame relative to transcription initiator and promoter sequences active in the plant or bacterium. Transcription and translation of the coding sequence under control of the regulatory sequences, can cause expression of the enzyme at levels which provide an effective amount of a monoterpene such as limonene in the tissue of the plant which are normally infested by the larvae.

As an illustration, it can be noted that Colby *et al.*, at the Keystone Symposium on Crop Improvement via Biotechnology: An International Perspective, Keystone, Colorado, USA, April 10-16, 1992, as reported in *J. Cell Biochem. Suppl. 16* F, 230 (1992), have isolated and characterized cDNA encoding limonene cyclase from spearmint. To isolate and study the gene(s) (sic) encoding limonene synthase and to produce enough of the enzyme for structural studies, they used standard methods to extract RNA from young leaves of *Mentha spicata* and constructed a cDNA library in λ ZAP XR (Stratagene) from poly (A) + RNA. They designed three degenerate oligonucleotides based on internal amino acid sequences obtained from Edman degradation of purified limonene synthase and screened 250,000 clones to identify six positive clones that hybridized to all three oligonucleotides. The resulting clones could be used in the methods of this invention which involve plant transformation. However, Colby *et al.* indicate no appreciation of the value of the enzyme in conferring resistance to insects in plants.

In certain plants, including maize, at least one additional gene encoding GPP synthase is required for generation of plants with resistance to insects. Due to the fact that natural levels of GPP are low in such plants, there may be inadequate amounts of GPP for limonene production in these plants when they are transformed solely with the limonene synthase gene. In contrast, GPP levels in other plant species, such as spearmint, is adequate for limonene production. In such species, some GPP is used to generate limonene, some to generate other metabolites. Because GPP is derived from a pathway that is common among plant

species, the introduction of the GPP synthase gene and at least one monoterpene synthase gene such as limonene synthase into plant species lacking GPP can generate transgenic plants capable of producing GPP, and a desired monoterpene at levels effective to confer resistance to insects.

In this manner, the invention encompasses transforming plants with nucleotide sequences encoding GPP synthase and limonene synthase for the generation of plants producing effective amounts of limonene; with nucleotide sequences encoding GPP synthase, limonene synthase and carveol synthase for the generation of plants producing effective amounts of carveol; and with nucleotide sequences encoding GPP synthase and S-linalool synthase for the generation of plants producing effective amounts of S-linalool.

In one embodiment, the plant which can be benefitted by this invention is preferably a plant susceptible to infestation and damage by the larvae of the genus Diabrotica or whose harvested material is subject to attack by larvae of that insect. A prime example is corn (*Zea mays*). Thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, particularly those susceptible to *Diabrotica spp.*, including, without limitation, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manicot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hemerocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browallia*, *Glycine*, *Lolium*, *Triticum*, and *Datura*.

Thus, the methods of the invention are useful to transform plants and create or enhance resistance to insects in plants. By resistance to insects is intended that the plant-insect interaction, preferably a plant-insect pest interaction, is reduced, ameliorated, prevented, or eliminated.

Insect pests include but are not limited to insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera. Insect pests of the invention for the major crops include: <u>Maize</u>: *Ostrinia nubilalis*,

European corn borer; *Agrotis ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, surgarcane borer; *Diabrotica virgifera*, western corn rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus spp.*, wireworms; *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn blot leafminer; *Anaphothrips obscrurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus urticae*, twospotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranea*, granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes*, *Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*; corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge, *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella*

fusca, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *zygogramma exclamationis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera murtfeldtiana*, sunflower seed midge; Cotton *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm; *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*, bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Franklinkiella fusca*, tobacco thrips; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia includens*, soybean looper; *Anticarsia gemmatalis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer, *Agrotis ipsilon*, black cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum hilare*, green stink bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*, seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestani*, strawberry spider mite; *Tetranychus urticae*, twospotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Delia platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Brevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, Flea beetle; *Mamestra configurata*, Bertha armyworm; *Plutella xylostella*, Diamond-back moth; *Delia* ssp., Root maggots.

Preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean, fiber crops, such as cotton, fruit crops, such as melons, and vegetable crops, including onion, pepper, tomato, cucumber, squash, carrot, crucifer (cabbage, broccoli, cauliflower), eggplant, spinach, potato and lettuce.

While compounds other than monoterpenes have some effective insecticidal activity at high concentrations in pure form, plant cell expression at such high concentrations is either not possible in a living plant cell system, or is not feasible if the commercially useful characteristics of the plant are to be preserved in terms of production of oils, starches, fibers, or other materials. Monoterpenes, on the other hand, are not directly expressed as the gene product, and the peptide or peptides which is or are expressed in the methods of this invention is an enzyme which can catalyze the synthesis of large amounts of a monoterpene(s) in the tissues of the transformed plant (e.g. limonene synthase), and in instances in which it is required, an enzyme which can catalyze the synthesis of large amounts of substrate for a downstream monoterpene synthase (e.g. GPP synthase).

The genes utilized in the invention, including GPP-, carveol, limonene-, and S-linalool synthase genes can be optimized for enhanced expression in plants of interest. See, for example, EPA0359472; WO91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498. In this manner, the genes can be synthesized utilizing plant-preferred condons. See, for example, Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, the disclosure of which is incorporated herein by reference. In this manner, synthetic genes can also be made based on the distribution of codons a particular host uses for a particular amino acid. Thus, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

The methods of the invention encompass utilizing naturally occurring nucleotide sequences encoding GPP synthase, and the monoterpene synthases limonene-, carveol- and S-linalool synthases; or utilizing synthetically derived

sequences encoding these proteins. The naturally occurring nucleotide sequences utilized in the methods of the invention are set forth in Figures 6, 8, 10 and 12.

The methods also encompass utilizing nucleotide sequences isolated from various organisms including plants by hybridization with partial sequences obtained from the natural sequences as set forth above. Conditions that will permit other DNA sequences to hybridize to the DNA sequences set forth herein can be determined in accordance with techniques generally known in the art. For example, hybridization of such sequences may be carried out under conditions of reduced stringency, medium stringency, or high stringency conditions (*e.g.*, conditions represented by a wash stringency of 35-40% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 37°C; conditions represented by a wash stringency of 40-45% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42°C; and conditions represented by a wash stringency of 50% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42°C, respectively. *See* Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). The methods further encompass utilizing nucleotide sequences isolated from various organisms including plants by other well known methods such as PCR using the natural sequences as set forth above.

The methods of the invention comprise utilizing expression cassettes with constitutive or tissue-specific promoters. Promoters that may be used in the expression cassettes include without limitation nos, ocs, phaseolin, FMV and other promoters isolated from the DNA of plants or other sources, both natural and synthetic.

Constitutive promoters would provide a constant production of the enzymes GPP-, limonene-, carveol and/or S-linalool synthase and thereby the corresponding monoterpene(s). Such constitutive promoters include, for example, the core promoter of the Rsyn7 (U.S. Patent Application Serial No. 08/661,601), the core CaMV 35S promoter (Odell *et al.* (1985) *Nature 313*:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18* 675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730); ALS promoter (U.S. Patent Application Serial

No. 08/409,297), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

An efficient plant promoter that may be used is an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-bisphosphate carboxylase from soybean (Berry-Lowe *et al.* (1982) *J. Mol. and App. Gen. 1*:483-498, and the promoter of the chlorophyll a-b binding protein. However, these two promoters are known to be light-induced in eukaryotic plant cells (see, for example, *Genetic Engineering of Plants, An Agricultural Perspective*, Cashmore, Pelham, New York, 1983, pp. 29-38, G. Coruzzi *et al.* (1983) *J. Biol. Chem. 258*:1399 and P. Dunsmuir *et al.* (1983) *J. Mol. and App. Gen. 2*:285 and may be less desirable when root expression is desired.

A tissue-specific promoter (or promoters) can be used in any instance where it may be desirable to localize production of the desired monoterpene to an insect-infested tissue or to a tissue which is efficient in production of a desired enzyme. The utilization of tissue-specific promoters would increase or decrease the expression of monoterpene synthases and production of the corresponding monoterpenes in specific tissues of the plant. It is recognized that in manipulating the level of monoterpene production as such, it may be desirable to increase or decrease the levels of such molecules in a particular tissue, since tolerance of various tissues to increased expression of specific monoterpenes may vary. Thus, it may be desirable to increase expression in selected tissues, or at varying levels in different tissues by the use of tissue-specific promoters.

Particular tissue-specific promoters of interest includes root-preferred promoters. The utilization of such promoters would provide a method of selectively creating or enhancing resistance to insects, and/or manipulating levels of monoterpene molecules in the root. Since corn rootworm attack roots, root-specific promoters are especially preferred for the control of corn rootworm, while minimizing limonene production in the agronomically valuable parts of the plant. Such selectivity could also be particularly desirable in plants in which the root constitutes the food crop, including, but not limited to carrot, potato, radish, and the like.

Root specific promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. For example, Hire, *et al.* (1992) *Plant Mol Biology, 20(2)*: 207-218, describe a root-specific glutamine synthetase gene from soybean. Keller and Baumgartner, (1991) *The Plant Cell, 3(10)*:1051-1061, describe a root-specific control element in the GRP 1.8 gene of French bean. Sanger *et al.* (1990) *Plant Mol. Biology, 14(3)*: 433-443, discuss the root-specific promoter of the Mannopine Synthase (MAS) gene of *Agrobacterium tumefaciens*. Miao *et al.* (1991) *The Plant Cell, 3(1)*:11-22, describe a full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean. Bogusz *et al.* (1990) *The Plant Cell, 2(7)*:633-641, discusses two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa*. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) *Plant Science (Limerick) 79(1)*:69-76, describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes*. They concluded that enhancer and tissue-specific DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) *EMBO Journal, 8(2)*:343-350, used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2′ gene was root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene. The TR1′ gene, fused to NPTII, (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster H *et al.* (1995) *Plant Mol. Biol. 29(4)*:759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol. 25(4)*:681-691. See also U.S. Patent Nos. 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

Other tissue-specific promoters include those described in Yamamoto *et al.* (1997) *Plant J. 12(2)*:255-265; Kawamata *et al.* (1997) *Plant Cell Physiol. 38(7)*:792-803; Hansen *et al.* (1997) *Mol. Gen. Genet. 254(3)*:337-343; Russell *et*

al. (1997) *Transgenic Res. 6(2)*:157-168; Rinehart *et al.* (1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Lam (1994) *Results Probl. Cell Differ. 20*:181-196; Orozco *et al.* (1993) *Plant Mol. Biol. 23(6)*:1129-1138; Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90(20)*:9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505.

Seed-preferred promoters includes both seed-specific promoters (those promoters active during seed development) as well as seed-germinating promoters (those promoters active during seed germination). Such promoters include Cim1 (cytokinin-induced message); cZ19B1 (maize 19KDa zein); mi1ps (myo-inositol-1-phosphate synthase); celA (cellulose synthase); end1 (*Hordeum verlgase* mRNA clone END1); and imp3 (myo-inositol monophosphate-3). For dicots, particular promoters include phaseolin, napin, β-conglycinin, soybean lectin, and the like. For monocots, particular promoters include maize 15Kd zein, 22KD zein, 27kD zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

In native tissues which synthesize monoterpenes, GPP-, limonene- and S-linalool synthases are targeted to plastids via plastid targeting sequences (transit peptides) typically contained at the N-terminus of the preprotein forms of the enzymes. The targeting sequences are then cleaved to release the mature enzymes in plastids. Such transit peptides can be identified in the primary amino acid sequences of the preproteins by those ordinarily skilled in the art. For example, see Colby *et al.* (1993) *J. Biol. Chem. 268(31)*:23016-23024, for the transit peptide sequence of limonene synthase. Native carveol synthase is associated with the endoplasmic reticulum, while native carveol dehydrogenase could be cytoplasmic.

Furthermore, the substrates of GPP synthase, IPP (IDP) and DMAPP (DMADP) can be found in plastids or in the cytoplasm (Bohlmann *et al.* (1998) *Proc. Natl. Acad. Sci. USA 95*:4126-4133). In plastids, these substrates are most likely synthesized via the glyceraldehyde-3-phosphate/pyruvate (Rohmer) pathway, and in the cytosol via the mevalonate pathway in the cytosol or the mevalonate pathway.

Thus, for manipulating a metabolic pathway involving monoterpene production, it would be beneficial to target GPP-, limonene-, and S-linalool

synthase to plastids; and carveol synthase and dehydrogenase to the endoplasmic reticulum (ER). This targeting could be achieved by use of the native targeting sequences contained in the sequences of the native proteins, or by addition or exchange of heterologous subcellular targeting signals. Alternatively, the enzymes utilized in the methods of the invention could be directed to the cytoplasm by deletion of the plastid and/or ER targeting signals. Methods for deletion, exchange and addition of nucleotide sequences are well known in the art, and can be readily used for manipulation of nucleotide segments encoding targeting signals of interest as described herein.

Heterologous sequences which can be used to target the desired enzymes of the invention to plastids include chloroplast targeting sequences. Chloroplast targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco), (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol. 30*:769-780; Schnell, *et al.* (1991) *J. Biol. Chem. 266(5)*:3335-3342), 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb. 22(6)*:789-810); tryptophan synthase (Zhao *et al.* (1995) *J.Biol. Chem. 270(11)*:6081-6087); plastocyanin (Lawrence *et al.* (1997) *J. Biol. Chem. 272(33)*:20357-20363); chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem. 268(36)*:27477-27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem. 263*:14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968, Romer *et al.* (1993) *Biochem. Biophys. Res Commun. 196*:1414-1421; and Shah *et al.* (1986) *Science 233*:478-481.

Likewise, methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:8526-8530; Svab and Maliga (1993) *Proc. Natl Acad. Sci. USA 90*:913-917; Staub and Maliga (1993) *Embo J. 12*:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-specific expression of a nuclear-encoded and plastid-directed RNA polymerase.

Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:7301-7305.

The nucleotide sequences utilized in methods of the invention can be introduced into any plant. The sequences to be introduced may be used in expression cassettes for expression in any plant of interest where expression in the plant is necessary for transcription.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of the enzymes of the invention in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

Where expression cassettes are needed, such expression cassettes will comprise a transcriptional initiation region linked to the coding sequence or antisense sequence of the nucleotide of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By foreign is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

The transcriptional cassette will include in the 5'-to-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell. 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-

158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; Joshi *et al.* (1987) *Nucleic Acids Res. 15*:9627-9639.

The methods of the invention encompass use of expression cassettes for expression of nucleotide sequences encoding GPP- and monoterpene synthases in the plant of interest. The cassette will include 5′ and 3′ regulatory sequences operably linked to the sequence of interest. The cassette may additionally contain at least one additional sequence to be cotransformed into the organism. Alternatively, the additional sequence(s) can be provided on another expression cassette.

Where appropriate, sequences utilized in the methods of the invention and additional gene(s) may be optimized for increased expression in the transformed plant. That is, these nucleotide sequences can be synthesized using plant preferred codons for improved expression. Methods are available in the art for synthesizing plant preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436, 391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences which may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Nat. Acad. Sci. USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986)); MDMV leader (Maize Dwarf Mosaic Virus) (*Virology 154*:9-20); human immunoglobulin heavy-chain binding protein (BiP) (Macejak and Sarnow (1991) *Nature 353*:90-94); untranslated leader from the coat protein mRNA of alfalfa

mosaic virus (AMV RNA 4) (Jobling and Gehrke (1987) *Nature 325*:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) *Molecular Biology of RNA*, pages 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also Della-Cioppa *et al.* (1987) *Plant Physiology 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.*, transitions and transversions, may be involved.

The methods of the invention can be used in transforming or transfecting any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.* monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Hinchee *et al.* (1988) *Biotechnology 6*:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes et al. (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Annual Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.*

27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (1995) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooydaas-Van Slogteren and Hooykaas (1984) *Nature (London) 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418; and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference. Thus, the invention provides a method for imparting insect resistance in *Agrobacterium tumefaciens*-susceptible plants in which the expression cassette is introduced into the cells by infecting the cells with *Agrobacterium tumefaciens*, a plasmid of which has been modified to include a plant expression cassette which expresses GPP- and/or a monoterpene synthase in the manner of this invention.

The modified plant may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell. Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting plants or hybrid plants the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

In a preferred embodiment, the invention provides methods of imparting resistance to *Diabrotica spp.* to plants of a susceptible taxon, comprising the steps of:

(a) culturing cells or tissues from at least one plant from the taxon,

(b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a structural gene coding for at least one monoterpene synthase or both a monoterpene synthase and GPP synthase, operably linked to a promoter that drives expression in a plant cell, and

(c) regenerating whole plants from the cell or tissue culture with resistance to insects. Once whole plants have been obtained, they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

Alternatively, once a single transformed plant has been obtained by the foregoing recombinant DNA method, conventional plant breeding methods can be used to transfer the structural gene or genes and associated regulatory sequences via crossing and backcrossing. Such intermediate methods will comprise the further steps of:

(a) sexually crossing the plant having resistance to insects with a plant from the taxon susceptible to insects;

(b) recovering reproductive material from the progeny of the cross; and

(c) growing plants having resistance to insects from the reproductive material. Where desirable or necessary, the agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to include the further steps of repetitively:

(a) backcrossing the progeny having resistance to insects with plants from taxon susceptible to insects; and

(b) selecting for the expression of resistance to insects (or an associated marker gene) or monoterpene production among the progeny of the backcross, until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with the gene imparting resistance to insects.

Insect pests of harvested material, including those of stored grain, can also be targets for the methods of this invention. In view of this, the invention also

provides methods for creating or enhancing resistance to insect pests in harvested materials and products obtained from harvested materials, by expressing at least one monotermene synthase and/or GPP synthase in the plant such that effective amounts of the desired monoterpene is produced in the harvested material and products obtained from such material.

The following examples are offered by way of illustration and not by way of limitation. The following description further exemplifies the compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

EXAMPLES 1-6

Insect larvae inhibition and toxicity assays for Southern Corn Rootworm (SCR) and European Corn Borer (ECB)

Bioassay diets were prepared as described in Czapla and Lang in "Effect of Plant Lectins on the Larval Development of European Corn Borer (Lepidoptera: Pyralidae) and Southern Corn Rootworm (Coleoptera: Chrysomelidae)", *J. Econ. Entomol. 83*:2480-85 (1990), except that low melting temperature agarose replaced the regular agarose so that the diets could be chilled to 37°C prior to the addition of limonene (one assay used the regular agarose diet).

Results were as follows. In Examples 1-4, the test larvae were Southern Corn Rootworm. In Examples 5-6, the test larvae were European Corn Borer. The results of each experiment represent the average from 16-32 insects. All limonene concentrations (ppm) are by weight. The SCR data indicate that limonene is effective against the larvae, but when limonene was used in the same protocol against ECB, little or no effect was seen.

$$\%\ \text{Corrected Mortality} = 100 \times \frac{(\text{mortality of treated} - \text{control})}{(100 - \text{control})}$$

$$\%\ \text{Wt. Reduction} = 100 \times \frac{(\text{control weight - treated weight})}{(\text{control wt})}$$

Example:	1		2	
Limonene ppm	% Corr. Mortal.	% weight Reduction	% Corr. Mortal.	% weight Reduction
10,000	57	64	80	86
1,000	49	52	19	30
100	57	52	15	6

Example:	3		4	
Limonene ppm	% Corr. Mortal.	% weight Reduction	% Corr. Mortal.	% weight Reduction
10,000	96	64	44	0
1,000	7	0	22	0
100	26	0	19	0

Average SCR Results:

Limonene ppm	% Corr. Mortal.	% weight Reduction
10,000	69	54
1,000	24	21
100	29	15

Example:	5		6	
Limonene ppm	% Corr. Mortal.	% weight Reduction	% Corr. Mortal.	% weight Reduction
10,000	0	28	0	0
1,000	0	16	0	0
100	0	1	0	7

Average ECB Results:

Limonene ppm	% Corr. Mortal.	% weight Reduction
10,000	0	14
1,000	0	8
100	0	4

EXAMPLE 7

Maize callus cultures were transformed by microprojectile bombardment using plasmids containing a cloned gene coding for the limonene synthase (limonene cyclase) enzyme driven by a ubiquitin promoter and a ubiquitin intron and followed downstream by a PIN-II terminator. Whole, fertile plants were regenerated from the transformed callus and analyzed for limonene synthase and limonene. Representative results from one series were as follows:

Clone #	Callus LS[1]	Callus Limonene2	Plant	Plant LS	Plant Limonene
C6	22539	ND	3	1950	ND
			7	2000	ND
C19	9900	ND	6	2100	ND
C3	2550	ND	3	1900	ND
			4	1650	ND

In other experiments, transgenic maize plants, and tissues that exhibited high expression of the limonene synthase protein were produced. The enzyme was extracted from transformed plants and tissues and allowed to react with tritium-labeled geranyl pyrophosphate (GPP). The extracted enzyme converted GPP to limonene, showing that a functional enzyme was being produced. In addition, western blots were done and confirmed the presence of LS protein in transgenic

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1 LS = Limonene Synthase

2 ND = not detected above 0.5 ng/g fresh wt.

tissues but not in negative control tissues. Seed was collected from the transformed plants. In sum, whole fertile, transformed plants have been produced that express active limonene cyclase (synthase) according to the teaching of the above-identified application.

Western blots were also performed on chloroplasts isolated from plants grown from T1 and T2 seed which expressed the mint limonene synthase gene (Figure 6) in leaf tissue. Thus, the mint limonene synthase was properly targeted to maize plastids and the protein was processed to the correct size, indicating that the mint limonene synthase plastid targeting sequence can be used to target proteins to maize plastids.

EXAMPLE 8

In view of the results set forth in Example 7, demonstrating expression of active limonene synthase in whole, fertile, transformed plants, larvicidally effective amounts of limonene can be produced in such transgenic plants where sufficient substrate is present for the limonene synthase enzyme to act on. Accordingly, maize callus cultures are transformed by art recognized microprojectile bombardment methods using plasmids containing genes coding for both the limonene synthase enzyme and the GPP synthase enzyme, driven, for example, by one or more promoters (a ubiquitin promoter, for example) and followed downstream by, for example, a PIN-II terminator. Whole, fertile plants are regenerated from the transformed callus and analyzed for presence of and/or activity of both enzymes, and are also analyzed for the presence of limonene.

Alternatively, callus is generated from transgenic plants that contain and express the limonene synthase transgene, and such callus cultures are transformed as described above, except that such callus is transformed using plasmids containing a gene coding for the GPP synthase protein. The whole, fertile, transgenic plants regenerated from such transformed callus produce larvicidally effective amounts of limonene.

EXAMPLE 9

Average minimum levels of various monoterpenes required for 100% mortality of Western corn rootworm were determined by the methods described in Examples 1-6 as follows:

Limonene-----200 ppm

S-linalool---50 ppm

carveol------25 ppm

EXAMPLE 10: Construction of Expression Vectors

Nucleotide sequences encoding limonene, GPP-, carveol and S-linalool synthase, as set forth in SEQ ID NOs: 1, 3, 5, and 7 respectively, are cloned into a plasmid vector, such as that shown in Figure 2, in the sense orientation so that they are under the transcriptional control of the ubiquitin promoter. A selectable marker gene may reside on this plasmid or may be introduced as part of a second plasmid. The transformation construct is then available for introduction into maize embryos by bombardment methods as described in Example 12.

EXAMPLE 11: Construction of Expression Vectors

Nucleotide sequences encoding GPP- and S-linalool synthase as described in EXAMPLE 10 were cloned in the sense orientation into an expression vector as shown in in Figure 3(PHP14099); such that both coding sequences are under the transcriptional control of the ubiquitin promoter. The plasmid contains the selectable marker gene PAT.

Nucleotide sequences encoding GPP-, limonene- and carveol synthases as described in EXAMPLE 10 were cloned in the sense orientation into an expression vector as shown in Figure 4(PHP14100); such that all three coding sequences are under the transcriptional control of the ubiquitin promoter. The plasmid contains the selectable marker gene PAT.

The transformation constructs PHP14099 and PHP14100 are available for introduction into maize embryos by bombardment methods as described in Example 12.

EXAMPLE 12: Transformation and Regeneration of Maize Callus

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the disclosed GPP- or monoterpene synthase gene operably linked to the ubiquitin promoter plus a plasmid containing the selectable marker gene PAT (Wohlleben et al. (1988) Gene 70:25-37) that confers resistance to the herbicide Bialophos. Transformation is performed as follows. All media recipes are in the Appendix.

Preparation of Target Tissue

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate. These are cultured on 560 L medium for 4 days prior to bombardment, in the dark. The day of bombardment, the embryos are transferred to 560 Y medium for 4 hours, arranged within the 2.5-cm target zone.

Preparation of DNA

A plasmid vector comprising the disclosed GPP- or monoterpene synthase operably linked to the ubiquitin promoter is constructed. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a CaCl2 precipitation procedure as follows:

100 μl prepared tungsten particles in water

10 μl (1 μg) DNA in TrisEDTA buffer (1 μg total)

100 μl 2.5 M $CaCl_2$

10 μl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is

removed, and 105 μl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 μl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialophos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are sampled for PCR and activity of the acetyl-CoA synthetase gene of interest. Positive lines are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored for expression of the GPP-synthase or monoterpene synthase gene of interest.

APPENDIX

Ingredient	Amount	Unit
D-I H2O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolystate (acid)	0.300	g
Sucrose	20.000	g
Glucose	0.600	g
2,4-D 0.5 mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1 mg/ml #	1.200	ml
Silver Nitrate 2 mg/ml #	1.700	ml

Directions:

@= Add after bringing up to volume

#= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

##= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$, $7H_2O$; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H_2O. Bring up to volume with D-I H_2O.

Total Volume (L) = 1.00

604 A

Ingredient	Amount	Unit
D-I H_2O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0 400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0 500	ml
L-Proline	1 980	g
Casein Hydrolysate (acid)	0 300	g
Sucrose	20.000	g
Glucose	0 600	g
2, 4-D 0.5mg/ml	1 600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml
Bialaphos 1 mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate, 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$,

$7H_2O$; and 28.300 g of Potassium Nitrate into sequence Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H_2O. Bring up to volume with D-I H_2O.

Total Volume (L) = 1.00

605 J

Ingredient	Amount	Unit
D-I H_2O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6 000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0 400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6 000	ml
Thiamine.HCL 0.4mg/ml	0 500	ml
Sucrose	20.000	g
Glucose	0 600	g
2, 4-D 0.5mg/ml	1 600	ml
Gelrite @	2 000	g
Dicamba 1mg/ml #	1 200	ml
Silver Nitrate 2mg/ml #	0 425	ml
Bialaphos 1 mg/ml #	3 000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$, $7H_2O$; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H_2O. Bring up to volume with D-I H_2O.

Total Volume (L) = 1.00

604 S

Ingredient	Amount	Unit
D-I H_2O	800.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolysate (acid)	0.300	g
Sucrose	120.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$, $7H_2O$; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in 950.000 ml of polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into 950.000 ml of D-I H_2O. Bring up to volume with D-I H_2O. Total Volume (L) = 1.00

272 V

Ingredient	Amount	Unit
D-I H_2O	950.000	ml
MS Salts (GIBCO 11117-074)	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Sucrose	40.000	g
Bacto-Agar @	6.000	g

Directions:

@ = Add after bringing up to volume

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occur, then make fresh stock.

Total Volume (L) = 1.00

288 J

Ingredient	Amount	Unit
D-I H_2O	950.000	ml
MS Salts	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Zeatin .5mg/ml	1.000	ml
Sucrose	60.000	g
Gelrite @	3.000	g
Indole Acetic Acid 0.5 mg/ml #	2.000	ml
.1mM Absissic Acid	1.000	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

Add 3.5g/L of Gelrite for cell biology.

= Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H_2O in sequence. Bring up to volume with polished D-I H2O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occur, then make fresh stock.

Total Volume (L) = 1.00

560 L

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	20.000	g
2, 4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

@ = Add after bringing up to volume

- Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H_2O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H_2O

Sterilize and cool to room temp.

Total Volume (L) = 1.00

560 R

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	30.000	g
2, 4-D 0.5mg/ml	4.000	ml
Gelrite @	3.000	g
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H_2O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H_2O

Sterilize and cool to room temp.

Total Volume (L) = 1.00

560 Y

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	120.000	g
2,4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H2O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H_2O

Sterilize and cool to room temp.

** Autoclave less time because of increased sucrose**

Total Volume (L) = 1.00

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method for manipulating a metabolic pathway in a plant cell, said method comprising transforming a plant cell with at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell, wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a GPP synthase protein,

c) a nucleotide sequence encoding a carveol synthase protein,

d) a nucleotide sequence encoding a S-linalool synthase protein,

e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,

g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

j) a nucleotide sequence encoding a GPP synthase protein as as set forth in SEQ ID NO:3,

k) a nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, j, k or l.

2. The method of claim 1 wherein said plant cell is monocotyledonous.

3. The method of claim 2 wherein said monocot cell is a corn, wheat, rice, oat, rye or sorghum cell.

4. The method of claim 1 wherein said promoter is a constitutive promoter.

5. The method of claim 4 wherein said constitutive promoter is a ubiquitin promoter.

6. The method of claim 1 wherein said promoter is a tissue-specific promoter.

7. 7.The method of claim 6 wherein said tissue-specific promoter is a root-specific promoter.

8. A method for manipulating a metabolic pathway in a plant cell, said method comprising transforming a plant cell with a first nucleotide sequence encoding a GPP synthase protein, and at least one nucleotide sequence which is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a carveol synthase protein,

c) a nucleotide sequence encoding a S-linalool synthase protein,

d) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

e) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

f) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

g) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

h) A nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

i) A nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

j) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h or i; and

wherein said first and said second nucleotide sequence are operably linked to a promoter that drives expression in a plant cell.

9. The method of claim 8, wherein said plant cell is co-transformed.

10. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein

said selected nucleotide sequence consists of a second nucleotide sequence encoding a limonene synthase protein, and

further comprising regenerating stably transformed plants producing effective amounts of limonene.

11. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein

said selected nucleotide sequences consist of a second nucleotide sequence encoding a limonene synthase protein and

a third nucleotide sequence encoding a carveol synthase protein; and

further comprising regenerating stably transformed plants producing effective amounts of carveol.

12. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein

said selected nucleotide sequence consists of a second nucleotide sequence encoding a S-linalool synthase protein, and

further comprising regenerating stably transformed plants producing effective amounts of S-linalool.

13. The method of any of the claims 10, 11, or 12 wherein said plant is monocotyledonous.

14. The method of claim 13 wherein said monocot plant is a corn plant (Z. mays L.).

15. The method of claim 14 wherein said insect is larvae of *Diabrotica spp.*

16. A method of producing limonene synthase and GPP synthase in a plant cell, said method comprising transforming a first plant cell with a nucleotide sequence encoding a limonene synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a GPP synthase protein operably linked to a promoter that drives expression in a plant cell,

regenerating a first fertile transgenic plant from said first plant cell,

regenerating a second fertile transgenic plant from said second plant cell,

crossing said first and second fertile transgenic plants; and

recovering progeny from said cross which express limonene synthase and GPP synthase.

17. A method of producing limonene synthase, GPP synthase, and carveol synthase in a plant cell, said method comprising

transforming a first plant cell with a nucleotide sequence encoding a limonene synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a GPP synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a third plant cell with a nucleotide sequence encoding a carveol synthase protein operably linked to a promoter that drives expression in a plant cell,

producing a first fertile transgenic plant from said first plant cell,

producing a second fertile transgenic plant from said second plant cell,

producing a third fertile transgenic plant from said third plant cell,

performing a first cross between said first and second fertile transgenic plants,

recovering progeny from said first cross,

performing a second cross between said third fertile transgenic plant and said progeny of said first cross; and

recovering progeny from said second cross which express limonene synthase, GPP synthase and carveol synthase.

18. The method of claim 17, wherein said second plant cell is transformed with a nucleotide sequence encoding a carveol synthase protein, and said third plant cell is transformed with a nucleotide sequence encoding GPP synthase.

19. A method of producing GPP synthase and S-linalool synthase in a plant cell, said method comprising

transforming a first plant cell with a nucleotide sequence encoding a GPP synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a S-linalool synthase protein operably linked to a promoter that drives expression in a plant cell,

producing a first fertile transgenic plant from said first plant cell,

producing a second fertile transgenic plant from said second plant cell,

crossing said first and second fertile transgenic plants; and

recovering progeny from said cross which express GPP synthase and S-linalool synthase.

20. A transformed plant cell having a manipulated metabolic pathway by having stably incorporated into its genome at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell,

wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a GPP synthase protein,

c) a nucleotide sequence encoding a carveol synthase protein,

d) a nucleotide sequence encoding a S-linalool synthase protein,

e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,

g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

j) a nucleotide sequence encoding a GPP synthase protein as as set forth in SEQ ID NO:3,

k) a nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, k or l.

21. A transformed plant having a manipulated metabolic pathway by having stably incorporated into its genome at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell,

wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a GPP synthase protein,

c) a nucleotide sequence encoding a carveol synthase protein,

d) a nucleotide sequence encoding a S-linalool synthase protein,

e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,

g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

j) a nucleotide sequence encoding a GPP synthase protein as as set forth in SEQ ID NO:3,

k) a nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, j, k or l.

22. The plant of claim 21 wherein said plant is monocotyledonous.

23. The plant of claim 22 wherein said monocot plant is a corn, wheat, rice, oat, rye or sorghum plant.

24. The plant of claim 21 wherein said promoter is a constitutive promoter.

25. The plant of claim 24 wherein said constitutive promoter is a ubiquitin promoter.

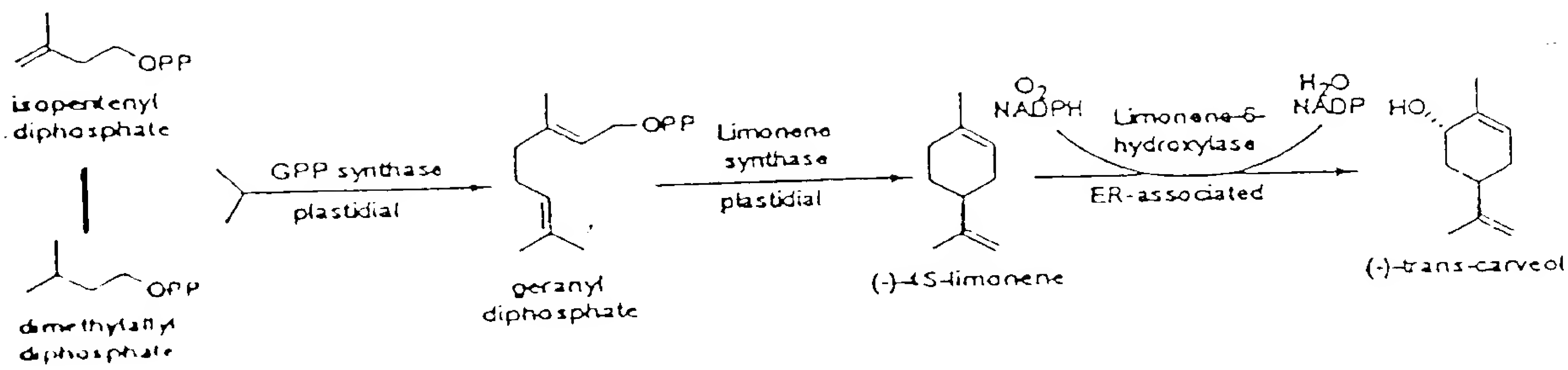
26. The plant of claim 21 wherein said promoter is a tissue-specific promoter.

27. The plant of claim 26 wherein said tissue-specific promoter is a root-specific promoter.

28. The plant of any of claims 24-27 wherein said plant is a dicot.

29. Seed of the plant of any of claims 21-27.

30. Seed of the plant of claim 28.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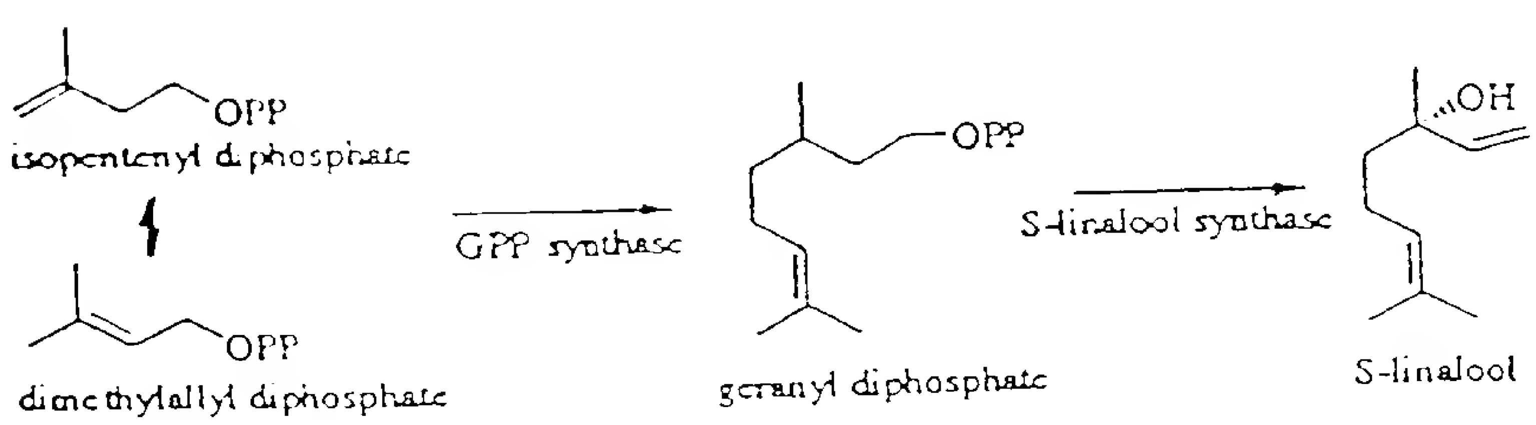
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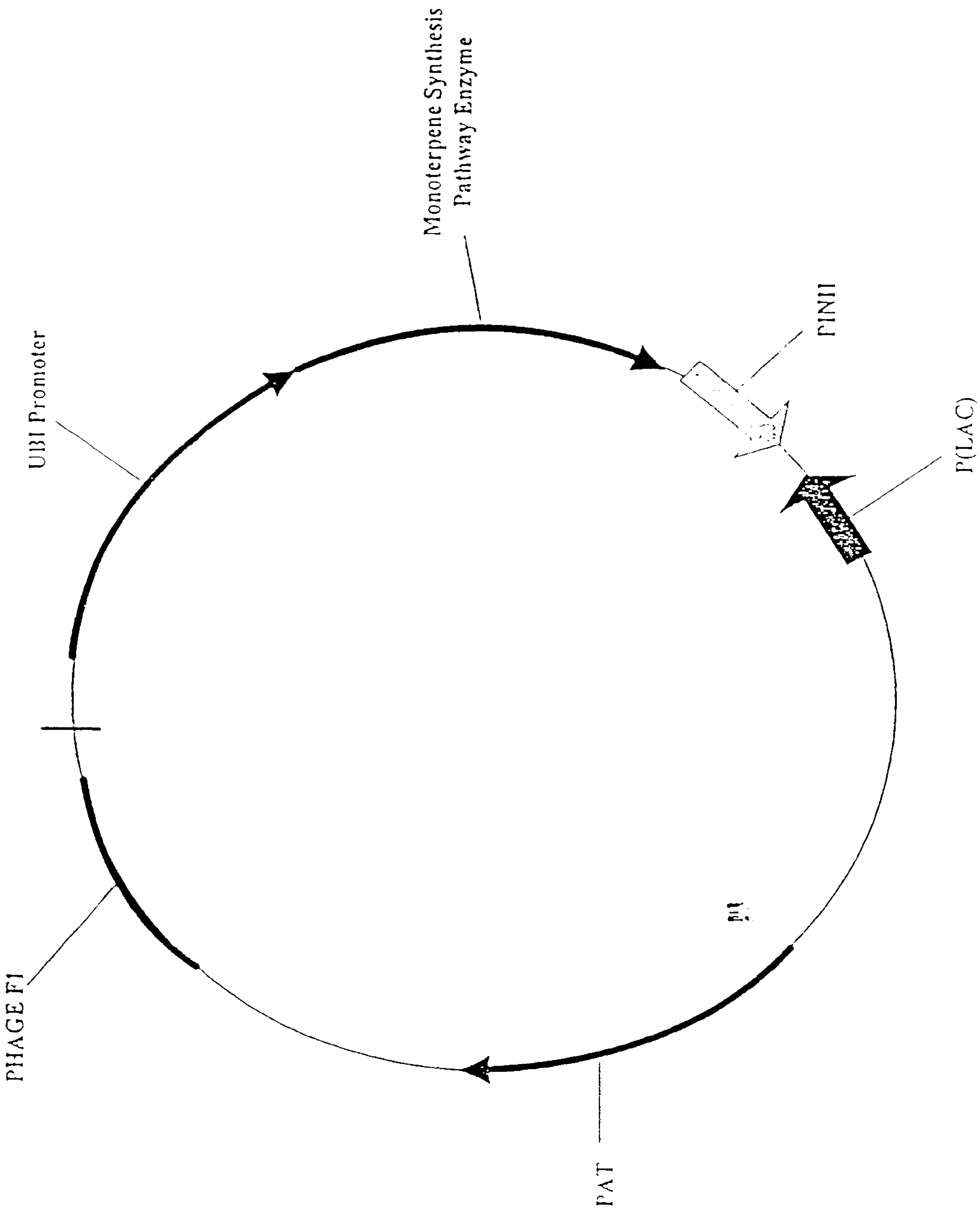
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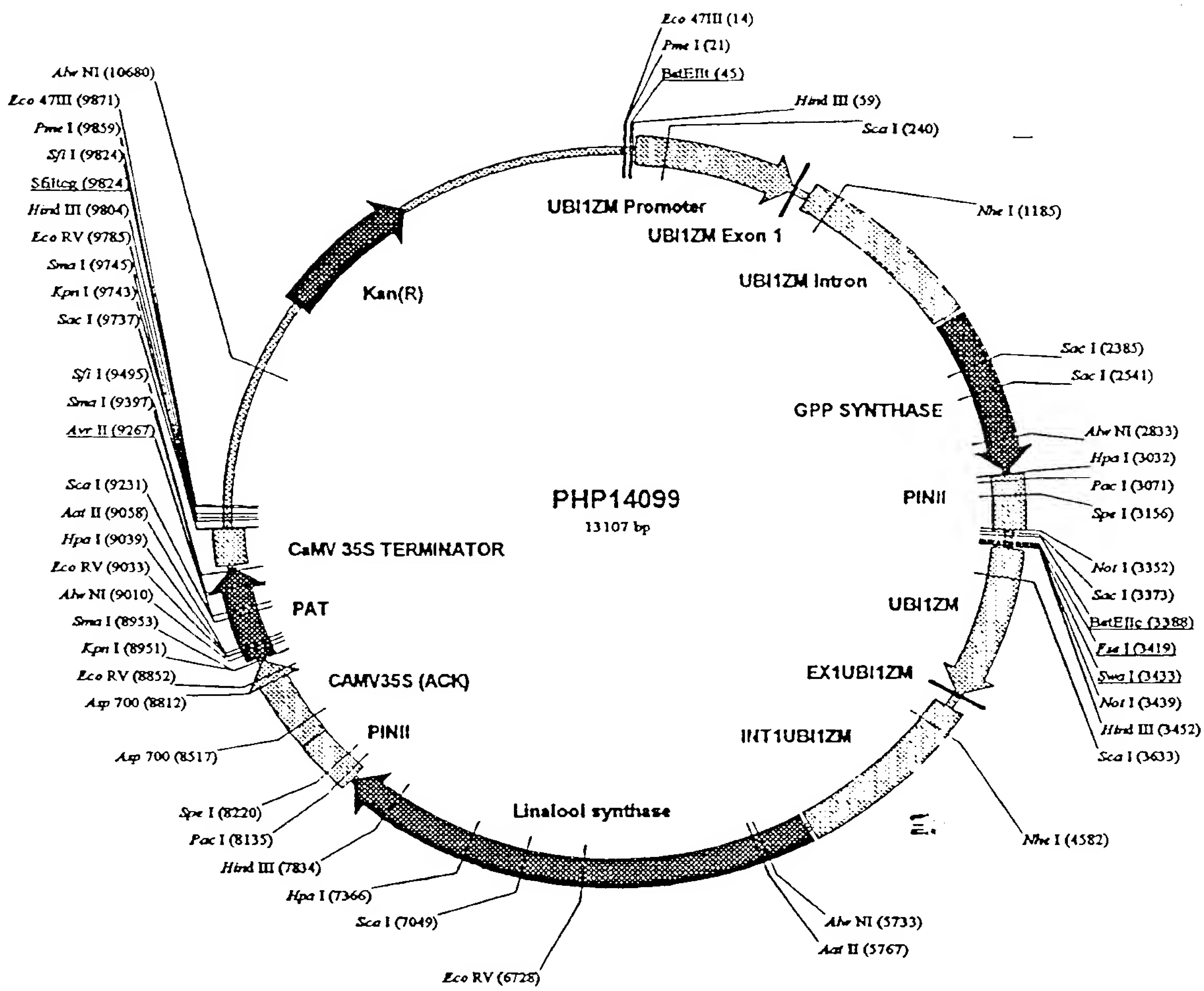
Figure 2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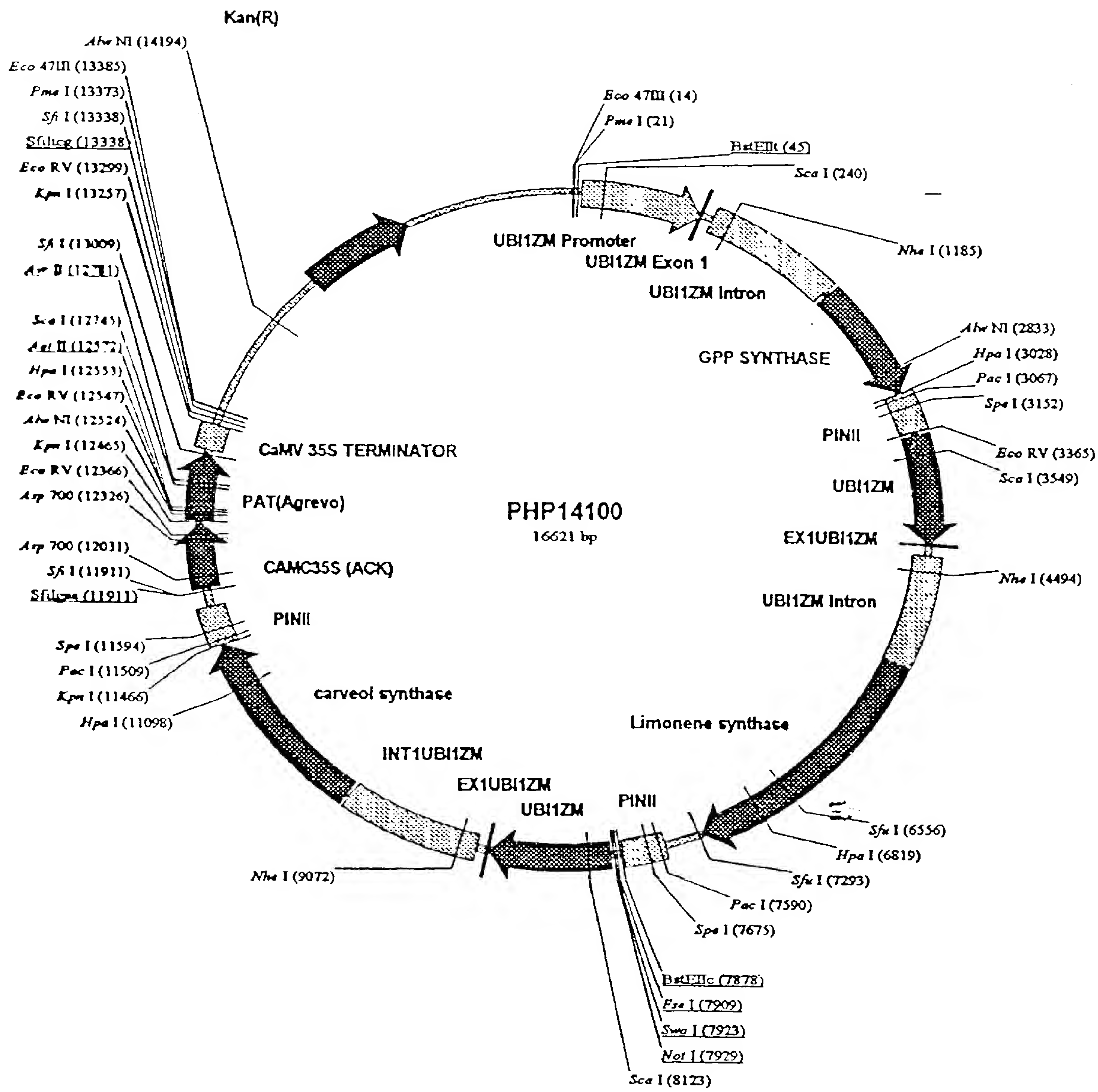
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Ser Leu Glu Glu Tyr Leu Glu Asn Ser Trp Gln Ser Ile Ser Gly Pro
445 450 455

tgt atg tta acg cac ata ttc ttc cga gta aca gat tcg ttc aca aag 1444
Cys Met Leu Thr His Ile Phe Phe Arg Val Thr Asp Ser Phe Thr Lys
460 465 470

gag acc gtc gac agt ttg tac aaa tac cac gat tta gtt cgt tgg tca 1492
Glu Thr Val Asp Ser Leu Tyr Lys Tyr His Asp Leu Val Arg Trp Ser
475 480 485

tcc ttc gtt ctg cgg ctt gct gat gat ttg gga acc tcg gtg gaa gag 1540
Ser Phe Val Leu Arg Leu Ala Asp Asp Leu Gly Thr Ser Val Glu Glu
490 495 500

gtg agc aga ggg gat gtg ccg aaa tca ctt cag tgc tac atg agt gac 1588

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Val Ser Arg Gly Asp Val Pro Lys Ser Leu Gln Cys Tyr Met Ser Asp
505                 510                 515                 520

tac aat gca tcg gag gcg gag gcg cgg aag cac gtg aaa tgg ctg ata      1636
Tyr Asn Ala Ser Glu Ala Glu Ala Arg Lys His Val Lys Trp Leu Ile
                525                 530                 535

gcg gag gtg tgg aag aag atg aat gcg gag agg gtg tcg aag gat tct      1684
Ala Glu Val Trp Lys Lys Met Asn Ala Glu Arg Val Ser Lys Asp Ser
            540                 545                 550

cca ttc ggc aaa gat ttt ata gga tgt gca gtt gat tta gga agg atg      1732
Pro Phe Gly Lys Asp Phe Ile Gly Cys Ala Val Asp Leu Gly Arg Met
        555                 560                 565

gcg cag ttg atg tac cat aat gga gat ggg cac ggc aca caa cac cct      1780
Ala Gln Leu Met Tyr His Asn Gly Asp Gly His Gly Thr Gln His Pro
    570                 575                 580

att ata cat caa caa atg acc aga acc tta ttc gag ccc ttt gca          1825
Ile Ile His Gln Gln Met Thr Arg Thr Leu Phe Glu Pro Phe Ala
585                 590                 595

tgagagatga tgacgagcca tcgtttactt acttaaattc taccaaagtt tttcgaaggc    1885
atagttcgta atttttcaag caccaataaa taaggagaat cggctcaaac aaacgtggca    1945
tttgccacca cgtgagcaca agggagagtc tgtcgtcgtt tatggatgaa ctattcaatt    2005
tttatgcatg taataattaa gttcaagttc aagagccttc tgcatattta actatgtatt    2065
tgaatttatc gagtgtgatt ttctgtcttt ggcaacatat atttttgtca tatgtggcat    2125
cttattatga tatcatacag tgtttatgga tgatatgata ctatc                    2170

      <210> 2
      <211> 599
      <212> PRT
      <213> Mentha spicata

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Met Ala Leu Lys Val Leu Ser Val Ala Thr Gln Met Ala Ile Pro Ser
 1               5                  10                  15
Asn Leu Thr Thr Cys Leu Gln Pro Ser His Phe Lys Ser Ser Pro Lys
            20                  25                  30
Leu Leu Ser Ser Thr Asn Ser Ser Ser Arg Ser Arg Leu Arg Val Tyr
        35                  40                  45
Cys Ser Ser Ser Gln Leu Thr Thr Glu Arg Arg Ser Gly Asn Tyr Asn
    50                  55                  60
Pro Ser Arg Trp Asp Val Asn Phe Ile Gln Ser Leu Leu Ser Asp Tyr
65                  70                  75                  80
Lys Glu Asp Lys His Val Ile Arg Ala Ser Glu Leu Val Thr Leu Val
                85                  90                  95
Lys Met Glu Leu Glu Lys Glu Thr Asp Gln Ile Arg Gln Leu Glu Leu
            100                 105                 110
Ile Asp Asp Leu Gln Arg Met Gly Leu Ser Asp His Phe Gln Asn Glu
        115                 120                 125
Phe Lys Glu Ile Leu Ser Ser Ile Tyr Leu Asp His His Tyr Tyr Lys
    130                 135                 140
Asn Pro Phe Pro Lys Glu Glu Arg Asp Leu Tyr Ser Thr Ser Leu Ala
145                 150                 155                 160
Phe Arg Leu Leu Arg Glu His Gly Phe Gln Val Ala Gln Glu Val Phe
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                    165                             170                 175
Asp Ser Phe Lys Asn Glu Glu Gly Glu Phe Lys Glu Ser Leu Ser Asp
                180                 185                 190
Asp Thr Arg Gly Leu Leu Gln Leu Tyr Glu Ala Ser Phe Leu Leu Thr
            195                 200                 205
Glu Gly Glu Thr Thr Leu Glu Ser Ala Arg Glu Phe Ala Thr Lys Phe
    210                 215                 220
Leu Glu Glu Lys Val Asn Glu Gly Gly Val Asp Gly Asp Leu Leu Thr
225                 230                 235                 240
Arg Ile Ala Tyr Ser Leu Asp Ile Pro Leu His Trp Arg Ile Lys Arg
                245                 250                 255
Pro Asn Ala Pro Val Trp Ile Glu Trp Tyr Arg Lys Arg Pro Asp Met
            260                 265                 270
Asn Pro Val Val Leu Glu Leu Ala Ile Leu Asp Leu Asn Ile Val Gln
        275                 280                 285
Ala Gln Phe Gln Glu Glu Leu Lys Glu Ser Phe Arg Trp Trp Arg Asn
    290                 295                 300
Thr Gly Phe Val Glu Lys Leu Pro Phe Ala Arg Asp Arg Leu Val Glu
305                 310                 315                 320
Cys Tyr Phe Trp Asn Thr Gly Ile Ile Glu Pro Arg Gln His Ala Ser
                325                 330                 335
Ala Arg Ile Met Met Gly Lys Val Asn Ala Leu Ile Thr Val Ile Asp
            340                 345                 350
Asp Ile Tyr Asp Val Tyr Gly Thr Leu Glu Glu Leu Glu Gln Phe Thr
        355                 360                 365
Asp Leu Ile Arg Arg Trp Asp Ile Asn Ser Ile Asp Gln Leu Pro Asp
    370                 375                 380
Tyr Met Gln Leu Cys Phe Leu Ala Leu Asn Asn Phe Val Asp Asp Thr
385                 390                 395                 400
Ser Tyr Asp Val Met Lys Glu Lys Gly Val Asn Val Ile Pro Tyr Leu
                405                 410                 415
Arg Gln Ser Trp Val Asp Leu Ala Asp Lys Tyr Met Val Glu Ala Arg
            420                 425                 430
Trp Phe Tyr Gly Gly His Lys Pro Ser Leu Glu Glu Tyr Leu Glu Asn
        435                 440                 445
Ser Trp Gln Ser Ile Ser Gly Pro Cys Met Leu Thr His Ile Phe Phe
    450                 455                 460
Arg Val Thr Asp Ser Phe Thr Lys Glu Thr Val Asp Ser Leu Tyr Lys
465                 470                 475                 480
Tyr His Asp Leu Val Arg Trp Ser Ser Phe Val Leu Arg Leu Ala Asp
                485                 490                 495
Asp Leu Gly Thr Ser Val Glu Glu Val Ser Arg Gly Asp Val Pro Lys
            500                 505                 510
Ser Leu Gln Cys Tyr Met Ser Asp Tyr Asn Ala Ser Glu Ala Glu Ala
        515                 520                 525
Arg Lys His Val Lys Trp Leu Ile Ala Glu Val Trp Lys Lys Met Asn
    530                 535                 540
Ala Glu Arg Val Ser Lys Asp Ser Pro Phe Gly Lys Asp Phe Ile Gly
545                 550                 555                 560
Cys Ala Val Asp Leu Gly Arg Met Ala Gln Leu Met Tyr His Asn Gly
                565                 570                 575
Asp Gly His Gly Thr Gln His Pro Ile Ile His Gln Gln Met Thr Arg
            580                 585                 590
Thr Leu Phe Glu Pro Phe Ala
        595
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<210> 3

<211> 1131
<212> DNA
<213> Mentha spicata

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<221> misc_feature
<222> (0)...(0)
<223> GPP Synthase

<221> CDS
<222> (6)...(944)

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Met Ala Ile Asn Leu Ser His Ile Asn Ser Lys Thr Cys Phe Pro
1 5 10 15

ctc aaa aca aga tct gat ctc agc cgt tct tct tcc gcg cgt tgc atg 98
Leu Lys Thr Arg Ser Asp Leu Ser Arg Ser Ser Ser Ala Arg Cys Met
20 25 30

cca act gcc gcc gct gcc gcc ttc ccc act atc gcc acc gcc gcc caa 146
Pro Thr Ala Ala Ala Ala Ala Phe Pro Thr Ile Ala Thr Ala Ala Gln
35 40 45

agt cag ccg tac tgg gcc gcc atc gag gcc gac ata gag aga tac ctg 194
Ser Gln Pro Tyr Trp Ala Ala Ile Glu Ala Asp Ile Glu Arg Tyr Leu
50 55 60

aag aaa tcc atc aca ata agg ccg ccg gag aca gtt ttc ggg ccc atg 242
Lys Lys Ser Ile Thr Ile Arg Pro Pro Glu Thr Val Phe Gly Pro Met
65 70 75

cac cac ctc acc ttc gcc gcc cca gcc acc gcc gcc tcc acc cta tgc 290
His His Leu Thr Phe Ala Ala Pro Ala Thr Ala Ala Ser Thr Leu Cys
80 85 90 95

ttg gcg gcg tgc gag ctc gtc ggc ggc gac cga agc caa gcc atg gca 338
Leu Ala Ala Cys Glu Leu Val Gly Gly Asp Arg Ser Gln Ala Met Ala
100 105 110

gcc gcg gcg gcg atc cat ctc gtg cac gcg gca gcc tac gtc cac gag 386
Ala Ala Ala Ala Ile His Leu Val His Ala Ala Ala Tyr Val His Glu
115 120 125

cac ctc cct cta acc gac ggg tcg agg ccc gta tcc aag ccc gca atc 434
His Leu Pro Leu Thr Asp Gly Ser Arg Pro Val Ser Lys Pro Ala Ile
130 135 140

cag cac aag tac ggc ccg aac gtc gag ctc ctc acc gga gac ggg att 482
Gln His Lys Tyr Gly Pro Asn Val Glu Leu Leu Thr Gly Asp Gly Ile
145 150 155

gtc ccg ttc ggg ttt gag ttg ctg gcc ggg tca gtg gac ccg gcc cga 530
Val Pro Phe Gly Phe Glu Leu Leu Ala Gly Ser Val Asp Pro Ala Arg
160 165 170 175

aca gac gac ccg gat agg att ctg aga gtt ata ata gag atc agt cgg 578
Thr Asp Asp Pro Asp Arg Ile Leu Arg Val Ile Ile Glu Ile Ser Arg
180 185 190

gcc ggc ggg ccg gag gga atg ata agc ggg ctg cat agg gaa gaa gaa 626
Ala Gly Gly Pro Glu Gly Met Ile Ser Gly Leu His Arg Glu Glu Glu
195 200 205

att gtt gat gga aat acg agt tta gac ttc att gaa tat gtg tgc aag 674
Ile Val Asp Gly Asn Thr Ser Leu Asp Phe Ile Glu Tyr Val Cys Lys
210 215 220

aaa aaa tac ggc gag atg cat gct tgc ggc gcg gct tgt gga gcc ata 722
Lys Lys Tyr Gly Glu Met His Ala Cys Gly Ala Ala Cys Gly Ala Ile
225 230 235

ttg ggc ggc gca gcc gag gag gag att cag aag ctg agg aat ttc ggg 770
Leu Gly Gly Ala Ala Glu Glu Glu Ile Gln Lys Leu Arg Asn Phe Gly
240 245 250 255

ctt tat caa gga act ctc aga gga atg atg gaa atg aaa aat tct cat 818
Leu Tyr Gln Gly Thr Leu Arg Gly Met Met Glu Met Lys Asn Ser His
260 265 270

caa tta att gat gag aat ata att gga aaa ttg aaa gaa ttg gct ctc 866
Gln Leu Ile Asp Glu Asn Ile Ile Gly Lys Leu Lys Glu Leu Ala Leu
275 280 285

gag gag ttg gga ggc ttc cac ggg aag aac gct gag ctg atg tcg agc 914
Glu Glu Leu Gly Gly Phe His Gly Lys Asn Ala Glu Leu Met Ser Ser
290 295 300

ctt gta gcc gag ccg agc ctt tac gcg gct tagagctatt cggatccttc 964
Leu Val Ala Glu Pro Ser Leu Tyr Ala Ala
305 310

attgcatttt catgcgacat cttcatattc atattgcata atatttttta agccagttat 1024
tttttatta tgaattttt taactgttat tgatttcgaa aatactgaca atcatctaaa 1084
ataaagtaaa tatagtaagg atgaaaaaa aaaaaaaaaa aaaaaaa 1131

<210> 4
<211> 313
<212> PRT
<213> Mentha spicata

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1 5 10 15
Lys Thr Arg Ser Asp Leu Ser Arg Ser Ser Ser Ala Arg Cys Met Pro
20 25 30
Thr Ala Ala Ala Ala Ala Phe Pro Thr Ile Ala Thr Ala Ala Gln Ser
35 40 45
Gln Pro Tyr Trp Ala Ala Ile Glu Ala Asp Ile Glu Arg Tyr Leu Lys
50 55 60
Lys Ser Ile Thr Ile Arg Pro Pro Glu Thr Val Phe Gly Pro Met His
65 70 75 80
His Leu Thr Phe Ala Ala Pro Ala Thr Ala Ala Ser Thr Leu Cys Leu

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                    85                                      90                                       95
Ala Ala Cys Glu Leu Val Gly Gly Asp Arg Ser Gln Ala Met Ala Ala
            100                 105                 110
Ala Ala Ala Ile His Leu Val His Ala Ala Ala Tyr Val His Glu His
        115                 120                 125
Leu Pro Leu Thr Asp Gly Ser Arg Pro Val Ser Lys Pro Ala Ile Gln
    130                 135                 140
His Lys Tyr Gly Pro Asn Val Glu Leu Leu Thr Gly Asp Gly Ile Val
145                 150                 155                 160
Pro Phe Gly Phe Glu Leu Leu Ala Gly Ser Val Asp Pro Ala Arg Thr
                165                 170                 175
Asp Asp Pro Asp Arg Ile Leu Arg Val Ile Ile Glu Ile Ser Arg Ala
            180                 185                 190
Gly Gly Pro Glu Gly Met Ile Ser Gly Leu His Arg Glu Glu Glu Ile
        195                 200                 205
Val Asp Gly Asn Thr Ser Leu Asp Phe Ile Glu Tyr Val Cys Lys Lys
    210                 215                 220
Lys Tyr Gly Glu Met His Ala Cys Gly Ala Ala Cys Gly Ala Ile Leu
225                 230                 235                 240
Gly Gly Ala Ala Glu Glu Glu Ile Gln Lys Leu Arg Asn Phe Gly Leu
                245                 250                 255
Tyr Gln Gly Thr Leu Arg Gly Met Met Glu Met Lys Asn Ser His Gln
            260                 265                 270
Leu Ile Asp Glu Asn Ile Ile Gly Lys Leu Lys Glu Leu Ala Leu Glu
        275                 280                 285
Glu Leu Gly Gly Phe His Gly Lys Asn Ala Glu Leu Met Ser Ser Leu
    290                 295                 300
Val Ala Glu Pro Ser Leu Tyr Ala Ala
305                 310

<210> 5
<211> 1762
<212> DNA
<213> Mentha spicata

<220>
<221> misc_feature
<222> (0)...(0)
<223> Carveol Synthase

<221> CDS
<222> (20)...(1507)

<400> 5
aaaaaacwaa aaagaaacw atg gag ctc gac ctt ttg tcg gca att ata atc      52
                     Met Glu Leu Asp Leu Leu Ser Ala Ile Ile Ile
                     1               5                   10

ctt gtg gca acc tac atc gta tcc ctc cta atc aac caa tgg cga aaa      100
Leu Val Ala Thr Tyr Ile Val Ser Leu Leu Ile Asn Gln Trp Arg Lys
            15                  20                  25

tcg aaa tcc caa caa aac cta cct ccg agc cct ccg aag ctg ccg gtg      148
Ser Lys Ser Gln Gln Asn Leu Pro Pro Ser Pro Pro Lys Leu Pro Val
        30                  35                  40

atc ggc cac ctc cac ttc ctg tgg gga ggg ctt ccc cag cac gtg ttt      196
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Ile Gly His Leu His Phe Leu Trp Gly Gly Leu Pro Gln His Val Phe
45 50 55

agg agc ata gcc cag aag tac ggg ccg gtg gcg cac gtg cag ctg gga 244
Arg Ser Ile Ala Gln Lys Tyr Gly Pro Val Ala His Val Gln Leu Gly
60 65 70 75

gaa gtg tac tcg gtg gtg ctg tcg tcg gcg gag gca gcg ccg cag gcg 292
Glu Val Tyr Ser Val Val Leu Ser Ser Ala Glu Ala Ala Pro Gln Ala
80 85 90

atg aag gtg ctg gac ccg aac ttc gcc gac cgg ttc gac ggc atc ggg 340
Met Lys Val Leu Asp Pro Asn Phe Ala Asp Arg Phe Asp Gly Ile Gly
95 100 105

tcc agg acc atg tgg tac gac aaa gat gac atc atc ttc agc cct tac 388
Ser Arg Thr Met Trp Tyr Asp Lys Asp Asp Ile Ile Phe Ser Pro Tyr
110 115 120

aac gat cac tgg cgc cag atg cgg agg atc tgc gtg aca gag ctg ctg 436
Asn Asp His Trp Arg Gln Met Arg Arg Ile Cys Val Thr Glu Leu Leu
125 130 135

agc ccg aag aac gtc agg tcc ttc ggg tac ata agg cag gag gag atc 484
Ser Pro Lys Asn Val Arg Ser Phe Gly Tyr Ile Arg Gln Glu Glu Ile
140 145 150 155

gag cgc ctc atc cgg ctg ctc ggg tcg tcg ggg gga gcg ccg gtc gac 532
Glu Arg Leu Ile Arg Leu Leu Gly Ser Ser Gly Gly Ala Pro Val Asp
160 165 170

gtg acg gag gag gtg tcg aag atg tcg tgt gtc gtc gtg tgc agg gcg 580
Val Thr Glu Glu Val Ser Lys Met Ser Cys Val Val Val Cys Arg Ala
175 180 185

gcg ttc ggg agt gtg ctc aag gac cag ggt tcg ttg gcg gag ttg gtg 628
Ala Phe Gly Ser Val Leu Lys Asp Gln Gly Ser Leu Ala Glu Leu Val
190 195 200

aag gag tcg ctg gca ttg gcg tcc ggg ttt gag ctg gcg gat ctc tac 676
Lys Glu Ser Leu Ala Leu Ala Ser Gly Phe Glu Leu Ala Asp Leu Tyr
205 210 215

cct tcc tca tgg ctc ctc aac ctg ctt agc ttg aac aag tac agg ttg 724
Pro Ser Ser Trp Leu Leu Asn Leu Leu Ser Leu Asn Lys Tyr Arg Leu
220 225 230 235

cag agg atg cgc cgc cgc ctc gat cac atc ctt gat ggg ttc ctg gag 772
Gln Arg Met Arg Arg Arg Leu Asp His Ile Leu Asp Gly Phe Leu Glu
240 245 250

gag cat agg gag aag aag agc ggc gac ttt gga ggc gag gac atc gtc 820
Glu His Arg Glu Lys Lys Ser Gly Asp Phe Gly Gly Glu Asp Ile Val
255 260 265

gac gtt ctt ttc agg atg cag ccg ggc agc gac agc aaa att ccc att 868
Asp Val Leu Phe Arg Met Gln Pro Gly Ser Asp Ser Lys Ile Pro Ile

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270 275 280

act tcc aat tgc atc aag ggt ttc att ttc gac acc ttc tcc gcg gga 916
Thr Ser Asn Cys Ile Lys Gly Phe Ile Phe Asp Thr Phe Ser Ala Gly
285 290 295

gct gaa acg tct tcg acg acc atc tca tgg gcg ttg tcg gaa ctg atg 964
Ala Glu Thr Ser Ser Thr Thr Ile Ser Trp Ala Leu Ser Glu Leu Met
300 305 310 315

agg aat ccg gcg aag atg gcc aag gtg cag gcg gag gta aga gag gcg 1012
Arg Asn Pro Ala Lys Met Ala Lys Val Gln Ala Glu Val Arg Glu Ala
320 325 330

ctc aag gga aag aca gtc gtg gat ttg agc gag gtg caa gag cta aaa 1060
Leu Lys Gly Lys Thr Val Val Asp Leu Ser Glu Val Gln Glu Leu Lys
335 340 345

tac ctg aga tcg gtg tta aag gag act ctg agg ctg cac cct ccc ttt 1108
Tyr Leu Arg Ser Val Leu Lys Glu Thr Leu Arg Leu His Pro Pro Phe
350 355 360

cca tta atc cca aga caa tcc agg gaa gaa tgc gag gtt aac ggg tac 1156
Pro Leu Ile Pro Arg Gln Ser Arg Glu Glu Cys Glu Val Asn Gly Tyr
365 370 375

acg att ccg gcc aaa act aga atc ttc atc aac gtc tgg gct atc gga 1204
Thr Ile Pro Ala Lys Thr Arg Ile Phe Ile Asn Val Trp Ala Ile Gly
380 385 390 395

agg gat ccc caa tac tgg gaa gat ccc gac acc ttc cgc cct gag aga 1252
Arg Asp Pro Gln Tyr Trp Glu Asp Pro Asp Thr Phe Arg Pro Glu Arg
400 405 410

ttc gat gag gtt tcc agg gat ttc atg gga aac gat ttc gag ttc atc 1300
Phe Asp Glu Val Ser Arg Asp Phe Met Gly Asn Asp Phe Glu Phe Ile
415 420 425

cca ttc ggg gcg ggt cga aga atc tgc ccc ggt tta cat ttc ggg ctg 1348
Pro Phe Gly Ala Gly Arg Arg Ile Cys Pro Gly Leu His Phe Gly Leu
430 435 440

gca aat gtt gag atc cca ttg gcg caa ctg ctc tac cac ttc gac tgg 1396
Ala Asn Val Glu Ile Pro Leu Ala Gln Leu Leu Tyr His Phe Asp Trp
445 450 455

aaa ttg cca caa gga atg act gat gcc gac ttg gca ctg acg gag acc 1444
Lys Leu Pro Gln Gly Met Thr Asp Ala Asp Leu Ala Leu Thr Glu Thr
460 465 470 475

cca ggt ctt tct ggg cca aaa aag aaa aat gtt tgc ttg gtt ccc aca 1492
Pro Gly Leu Ser Gly Pro Lys Lys Lys Asn Val Cys Leu Val Pro Thr
480 485 490

ctc tat aaa agt cct taaccactaa gaagttagca taataagaca tctaaaattg 1547
Leu Tyr Lys Ser Pro
495
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tcataatcat ctaattattg ttacacttct tctatcatgt cattttgaga agtgtcttat 1607
agaggtggcc acggttccgg ttccagttcg gaagcggaac cgaaccatca gttacggttc 1667
tcagcaagaa gcgaaccgtc ccgccccccc tactgtgttt gagatataaa acacataaaa 1727
taaaataaaa aaaacgctat tttttttaa aaaaa 1762

<210> 6
<211> 496
<212> PRT
<213> Mentha spicata

<400> 6
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1 5 10 15
Ile Val Ser Leu Leu Ile Asn Gln Trp Arg Lys Ser Lys Ser Gln Gln
20 25 30
Asn Leu Pro Pro Ser Pro Pro Lys Leu Pro Val Ile Gly His Leu His
35 40 45
Phe Leu Trp Gly Gly Leu Pro Gln His Val Phe Arg Ser Ile Ala Gln
50 55 60
Lys Tyr Gly Pro Val Ala His Val Gln Leu Gly Glu Val Tyr Ser Val
65 70 75 80
Val Leu Ser Ser Ala Glu Ala Ala Pro Gln Ala Met Lys Val Leu Asp
85 90 95
Pro Asn Phe Ala Asp Arg Phe Asp Gly Ile Gly Ser Arg Thr Met Trp
100 105 110
Tyr Asp Lys Asp Asp Ile Ile Phe Ser Pro Tyr Asn Asp His Trp Arg
115 120 125
Gln Met Arg Arg Ile Cys Val Thr Glu Leu Leu Ser Pro Lys Asn Val
130 135 140
Arg Ser Phe Gly Tyr Ile Arg Gln Glu Glu Ile Glu Arg Leu Ile Arg
145 150 155 160
Leu Leu Gly Ser Ser Gly Gly Ala Pro Val Asp Val Thr Glu Glu Val
165 170 175
Ser Lys Met Ser Cys Val Val Val Cys Arg Ala Ala Phe Gly Ser Val
180 185 190
Leu Lys Asp Gln Gly Ser Leu Ala Glu Leu Val Lys Glu Ser Leu Ala
195 200 205
Leu Ala Ser Gly Phe Glu Leu Ala Asp Leu Tyr Pro Ser Ser Trp Leu
210 215 220
Leu Asn Leu Leu Ser Leu Asn Lys Tyr Arg Leu Gln Arg Met Arg Arg
225 230 235 240
Arg Leu Asp His Ile Leu Asp Gly Phe Leu Glu Glu His Arg Glu Lys
245 250 255
Lys Ser Gly Asp Phe Gly Gly Glu Asp Ile Val Asp Val Leu Phe Arg
260 265 270
Met Gln Pro Gly Ser Asp Ser Lys Ile Pro Ile Thr Ser Asn Cys Ile
275 280 285
Lys Gly Phe Ile Phe Asp Thr Phe Ser Ala Gly Ala Glu Thr Ser Ser
290 295 300
Thr Thr Ile Ser Trp Ala Leu Ser Glu Leu Met Arg Asn Pro Ala Lys
305 310 315 320
Met Ala Lys Val Gln Ala Glu Val Arg Glu Ala Leu Lys Gly Lys Thr
325 330 335
Val Val Asp Leu Ser Glu Val Gln Glu Leu Lys Tyr Leu Arg Ser Val
340 345 350
Leu Lys Glu Thr Leu Arg Leu His Pro Pro Phe Pro Leu Ile Pro Arg

355 360 365
Gln Ser Arg Glu Glu Cys Glu Val Asn Gly Tyr Thr Ile Pro Ala Lys
370 375 380
Thr Arg Ile Phe Ile Asn Val Trp Ala Ile Gly Arg Asp Pro Gln Tyr
385 390 395 400
Trp Glu Asp Pro Asp Thr Phe Arg Pro Glu Arg Phe Asp Glu Val Ser
405 410 415
Arg Asp Phe Met Gly Asn Asp Phe Glu Phe Ile Pro Phe Gly Ala Gly
420 425 430
Arg Arg Ile Cys Pro Gly Leu His Phe Gly Leu Ala Asn Val Glu Ile
435 440 445
Pro Leu Ala Gln Leu Leu Tyr His Phe Asp Trp Lys Leu Pro Gln Gly
450 455 460
Met Thr Asp Ala Asp Leu Ala Leu Thr Glu Thr Pro Gly Leu Ser Gly
465 470 475 480
Pro Lys Lys Lys Asn Val Cys Leu Val Pro Thr Leu Tyr Lys Ser Pro
485 490 495

<210> 7
<211> 2681
<212> DNA
<213> Clarkia breweri

<220>
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<222> (0)...(0)
<223> S-Linalool Synthase

<221> CDS
<222> (28)...(2637)

<400> 7
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Met Gln Leu Ile Thr Asn Phe Ser Ser
1 5

tca tca tca gaa ttg cag ttt ctt gtg gat aag gtt aag aga gaa tca 102
Ser Ser Ser Glu Leu Gln Phe Leu Val Asp Lys Val Lys Arg Glu Ser
10 15 20 25

ttg tct tct tca tca tct aat act cag aat ttg ttt ctc tca act tca 150
Leu Ser Ser Ser Ser Ser Asn Thr Gln Asn Leu Phe Leu Ser Thr Ser
30 35 40

cct tat gac act gct tgg ctc gcc ctt atc cct cat cct cat cat cac 198
Pro Tyr Asp Thr Ala Trp Leu Ala Leu Ile Pro His Pro His His His
45 50 55

cat cac cat ggc cga ccc atg ttt gaa aaa tgt ctg caa tgg att ctc 246
His His His Gly Arg Pro Met Phe Glu Lys Cys Leu Gln Trp Ile Leu
60 65 70

cat aac cag aca cca caa ggt ttc tgg gca gca gct ggt gac aat att 294
His Asn Gln Thr Pro Gln Gly Phe Trp Ala Ala Ala Gly Asp Asn Ile
75 80 85

tcc gac acc gac gat gac gtc acc ctg gat tgt ctt cta tca acc ttg 342

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Ser Asp Thr Asp Asp Asp Val Thr Leu Asp Cys Leu Leu Ser Thr Leu
 90                  95                 100                 105

gct tgc tta gtt gca ctc aaa agg tgg cag ctt gct ccc gac atg att      390
Ala Cys Leu Val Ala Leu Lys Arg Trp Gln Leu Ala Pro Asp Met Ile
                110                 115                 120

cat aaa gga ttg gaa ttt gta aat aga aac aca gag aga ctt gta atg      438
His Lys Gly Leu Glu Phe Val Asn Arg Asn Thr Glu Arg Leu Val Met
            125                 130                 135

aag cag aag ccg agc gac gtt cct cgt tgg ttc acc atc atg ttc ccg      486
Lys Gln Lys Pro Ser Asp Val Pro Arg Trp Phe Thr Ile Met Phe Pro
        140                 145                 150

gcg atg ctc gag ctt gcc gga gct tcc agt ctc cga gtc gat ttc agc      534
Ala Met Leu Glu Leu Ala Gly Ala Ser Ser Leu Arg Val Asp Phe Ser
    155                 160                 165

gag aat ctt aac aga atc ttg gtg gaa cta tct caa aat agg gat gat      582
Glu Asn Leu Asn Arg Ile Leu Val Glu Leu Ser Gln Asn Arg Asp Asp
170                 175                 180                 185

att ctc aca agg gag gaa gtt gat gag aag aag caa tac tca cca ttg      630
Ile Leu Thr Arg Glu Glu Val Asp Glu Lys Lys Gln Tyr Ser Pro Leu
                190                 195                 200

cta cta ttt cta gaa gca ttg cct gca caa tcc tat gac aat gat gtt      678
Leu Leu Phe Leu Glu Ala Leu Pro Ala Gln Ser Tyr Asp Asn Asp Val
            205                 210                 215

cta aag caa att ata gac aag aac ttg agc aat gat ggt tct tta ttg      726
Leu Lys Gln Ile Ile Asp Lys Asn Leu Ser Asn Asp Gly Ser Leu Leu
        220                 225                 230

caa tcg cct tct gct aca gca aga gca tac atg ata aca gga aat acc      774
Gln Ser Pro Ser Ala Thr Ala Arg Ala Tyr Met Ile Thr Gly Asn Thr
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acc atg gtc ctt aca gca tcc tgt ctt cta ggt ccc ggt ttc ccg gtt     2022
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Ala Leu Ile Pro His Pro His His His His His His Gly Arg Pro Met
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Phe Glu Lys Cys Leu Gln Trp Ile Leu His Asn Gln Thr Pro Gln Gly
65 70 75 80
Phe Trp Ala Ala Ala Gly Asp Asn Ile Ser Asp Thr Asp Asp Asp Val
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Thr Leu Asp Cys Leu Leu Ser Thr Leu Ala Cys Leu Val Ala Leu Lys
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Arg Trp Gln Leu Ala Pro Asp Met Ile His Lys Gly Leu Glu Phe Val
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Pro Arg Trp Phe Thr Ile Met Phe Pro Ala Met Leu Glu Leu Ala Gly
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225 230 235 240
Arg Ala Tyr Met Ile Thr Gly Asn Thr Arg Cys Leu Ser Tyr Leu His
245 250 255
Ser Leu Thr Asn Ser Cys Ser Asn Gly Gly Val Pro Ser Phe Tyr Pro
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Tyr Val Trp Met Tyr Met Ile Glu Asn Asn Gln Ala Ser Ile Asp Asp
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Tyr His His Pro Phe Lys Val Phe Thr Leu Gln Lys Lys Gln Ser Ser
        835                 840                 845
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Ile Lys Leu Cys Phe Ser
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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/82, 9/88, 9/10, 9/00, 15/11, A01H 5/00	A3	(11) International Publication Number: WO 00/22150 (43) International Publication Date: 20 April 2000 (20.04.00)

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US 09/172,339 (CON)
Filed on 14 October 1998 (14.10.98)

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MEYER, Terry, EuClaire [US/US]; 4338 101st Street, Urbandale, IA 50322 (US). YALPANI, Nasser [CA/US]; 6041 North Winwood Drive, Johnston, IA 50131 (US).

(74) Agents: SPRUILL, W., Murray et al.; Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234-4009 (US).

(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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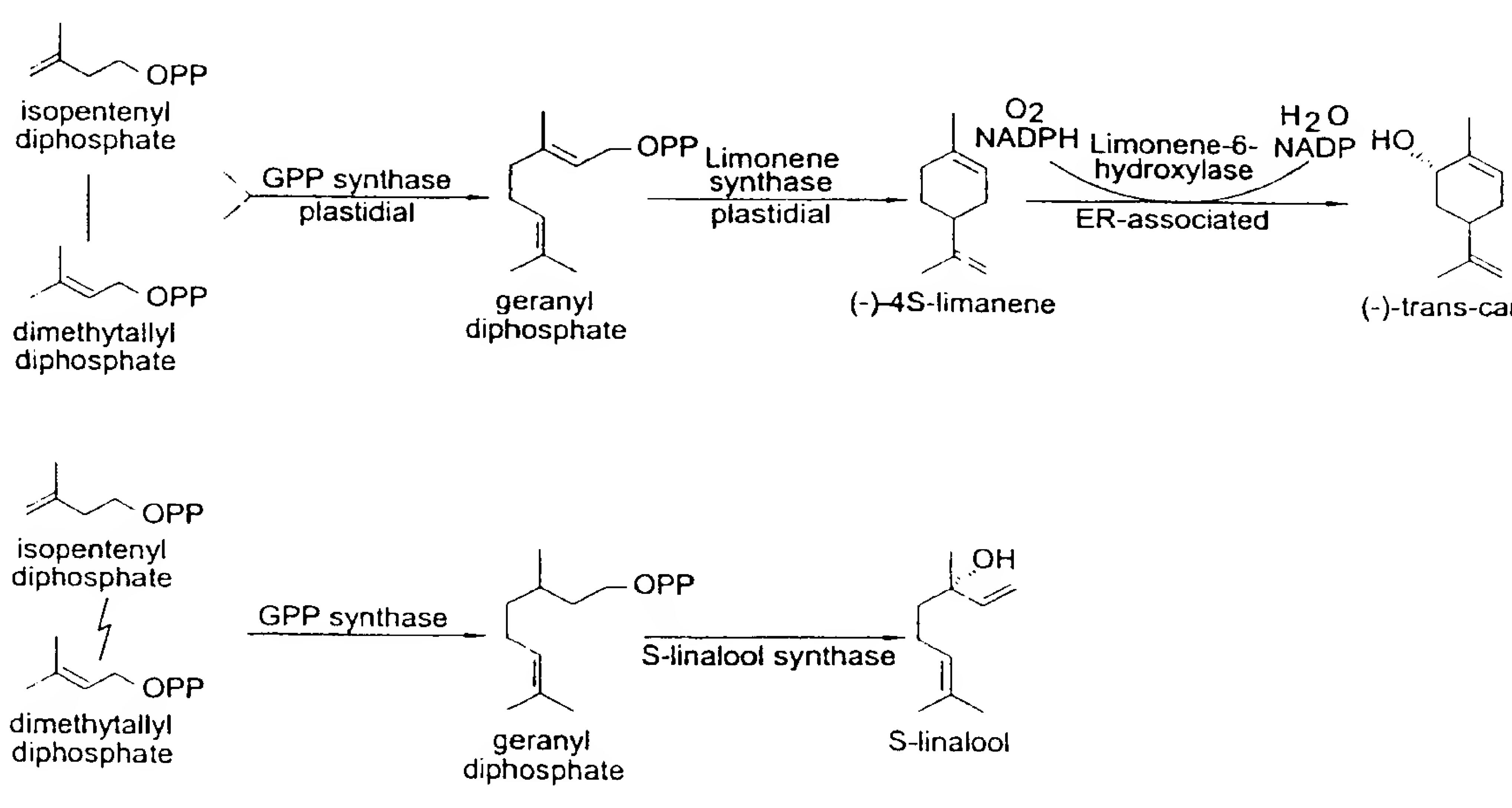
(54) Title: LIMONENE AND OTHER METABOLITES OF GERANYL PYROPHOSPHATE FOR INSECT CONTROL



(57) Abstract

Methods for manipulating metabolic pathway in plants, particularly those pathways involved in the biosynthesis of monoterpenes are provided. Methods are directed at transforming plants with one or more nucleotide sequences encoding the enzyme GPP synthase, and the monoterpene synthases limonene-, carveol and S-linolool synthase. Methods for creating or enhancing resistance to insects in plants by transforming plants with GPP- and/or monoterpene synthases, to generate plants producing monoterpenes in amount effective for resistance to insects are also provided.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/23180

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N9/88 C12N9/10 C12N9/00 C12N15/11
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37102 A (PIONEER HI BRED INT) 28 November 1996 (1996-11-28) the whole document ---	1-11, 13-16, 20-30
X	WO 94 22304 A (PIONEER HI BRED INT) 13 October 1994 (1994-10-13) the whole document ---	1-7, 20-30
X	WO 95 11913 A (UNIV WASHINGTON) 4 May 1995 (1995-05-04) the whole document ---	1-7, 20-30

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2000	25.07.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COLBY S M ET AL: "4S-LIMONENE SYNTHASE FROM THE OIL GLANDS OF SPEARMINT (MENTHA SPICATA)" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 268, no. 31, 5 November 1993 (1993-11-05), pages 23016-23024, XP002914702 ISSN: 0021-9258 the whole document -----	

1

Inte onal application No.
PCT/US 99/23180

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

SEE ADDITIONAL SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11, 13-18, 20-30 - partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: {1-11, 13-18, 20-30 partially}

   A method for manipulating a metabolic pathway in a plant cell comprising transforming a plant cell with a limonene synthase-encoding nucleotide sequence set forth in SEQ ID NO: 1 and coding for a protein set forth in SEQ ID NO: 2. A method for creating or enhancing resistance to insects in a plant, a method for producing limonene synthase in a plant cell, a transformed plant cell and plant having a manipulated metabolic pathway comprising said limonene synthase.

2. Claims: {1-30 partially}

   same as invention 1 but comprising a GPP synthase as set forth in SEQ ID NO: 3 and 4.

3. Claims: {1-9, 11, 13-15, 17, 18, 20-30 partially}

   same as in invention but comprising a carveol synthase as set forth in SEQ ID NO: 5 and 6.

4. Claims: {1-9, 12-15, 19-30 partially}

   same as invention 1 but comprising an S-linalool synthase as set forth in SEQ ID NO: 7 and 8.

INTERNATIONAL SEARCH REPORT

Info.   .tion on patent family members

Internatic   Application No

PCT/US 99/23180

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9637102 A	28-11-1996	AT 185043 T	15-10-1999
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